1988

Interaction of enteroluminal neutrophils and the Escherichia coli heat-stable enterotoxin b with porcine small intestine

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Order Number 8825443

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Iowa State University, 1988

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Interaction of enteroluminal neutrophils and the *Escherichia coli* heat-stable enterotoxin b with porcine small intestine

by

Rebecca Rose

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY
Major: Veterinary Pathology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa
1988

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GENERAL INTRODUCTION

The neutrophil is a cell which can phagocytose and kill pathogens and which can also secrete substances through degranulation. Neutrophils participate readily in nonspecific immune reactions. They also have a role in specific immune reactions; phagocytosis is enhanced by specific antibody. The mucosal surface of the intestine is certainly a major site of interaction between the body and the external environment. Neutrophils are an important "first line of defense" on other body surfaces, and it is logical to think that they might play an important role in protection of mucosal surfaces. Of course neutrophils would be part of an inflammatory response within the intestinal wall following tissue injury such as that due to invasive bacteria, but neutrophils might also serve a protective role within the lumen of the intestine without concomitant inflammation within the intestinal wall.

Infection of the swine small intestine with enterotoxigenic Escherichia coli is a good system to use for the study of neutrophils within the intestinal lumen. These pathogenic bacteria are found only in the lumen. They do not invade the intestinal tissue, and historically they have been thought to affect function of enterocytes without causing cellular degeneration or necrosis. Attraction of neutrophils into the porcine small intestine by enterotoxigenic E. coli has been described previously [107]. The mechanism by which these luminal bacteria attract neutrophils has not been described. The two major virulence factors of

these enterotoxigenic E. coli are their adhesins and their enterotoxins. Either adhesion to enterocytes by E. coli or elaboration of enterotoxin might be associated with attraction of neutrophils into the intestinal lumen.

Just as the role of enterotoxins in attracting enteroluminal neutrophils has not been described, neither have the effects of enterotoxins on small intestinal mucosa been clearly characterized. Mechanisms by which the E. coli heat-labile enterotoxin and the E. coli heat-stable enterotoxin a cause intestinal secretion are being defined [25,27,76]. A complete description of effects of the E. coli heat-stable enterotoxin b (STb) is lacking. Even the morphological effects of STb have been disputed. Some investigators have maintained that STb has no morphological effects [52]; others have suspected that STb could cause partial villous atrophy [102].

The objectives of the research presented here were i) to investigate the morphologic effects of STb on swine small intestinal mucosa, both at the light and electron microscopic level and ii) to examine bacterial factors which might be crucial in attraction of neutrophils into the intestinal lumen.

This dissertation is presented in alternate format including 2 manuscripts which have been published in scientific journals. The first manuscript has been published in Veterinary Pathology (Vet. Pathol. 24:71-79, 1987). The second manuscript has been published in Infection and Immunity (Infect. Immun. 48:818-823, 1985). The format used for

literature cited is that of the journal, Infection and Immunity. A literature review precedes the two papers, and a general summary and discussion follows the two papers. The literature cited within each paper is presented at the end of that paper. Literature cited within the introduction, literature review, and general summary and discussion is presented following the general summary and discussion.

The Ph.D. candidate, Rebecca Rose, was the principal or sole investigator for each of these studies.

LITERATURE REVIEW

The main topics which need to be examined are enteroluminal neutrophils of swine and Escherichia coli enterotoxins. Additional pertinent subjects are E. coli clinical syndromes, neutrophil chemotaxis, and exudate neutrophils.

Enteroluminal Neutrophils of Swine

When the statement is made that granulocytes are eliminated from the body through the intestine, two groups of researchers are often cited: Teir, Rytomaa, and Cederberg, and Ambrus and Ambrus.

The work of the first group [81,93,94] contains careful descriptions which today lead one to believe that they were describing interepithelial leukocytes rather than emigrating neutrophils. A careful reading of their work reveals numerous other instances of assumptions and over-interpretations.

Ambrus and Ambrus [1] used vascular and lymphatic catheterizations, intestinal fistulas, Thiry-Vella loops, and labelled leukocytes (labels were quinacrine and ^{32}P) to study the distribution of leukocytes in dogs. They concluded that granulocytes were sequestered in the lungs normally. (Today we might wonder if they had unintentionally activated C5a, which causes sequestration of neutrophils in the lung [70].) Although leukocytes were shown to be eliminated in the intestine, the majority of these were mononuclear cells.

After a brief review of some of this early literature, it is apparent why Patrick Murphy [64] began his chapter titled "Tissue Consumption of Granulocytes and Control Mechanisms for Granulopoiesis" with this statement:

> The reader in search of hard scientific information would do well to skip this chapter altogether, because it is constructed from a few wattles of established fact and large quantities of speculative daub.

In a review [21] written over 10 years after the work by Teir and Rytomaa and by Ambrus and Ambrus, work is cited which indicates that there are no pulmonary extravascular granulocytes found in pathogen-free mice although it is easy to find migrating granulocytes after induction of inflammation. The unpublished work of two other investigators was cited; they found no granulocytes in gut washings of rats and mice.

A recent review article [97] states that neutrophils in the peripheral blood are removed by splenic macrophages upon senescence. Neutrophils which have entered the tissues can be phagocytosed by monocytes or macrophages or leave the body by excretion from mucosal surfaces. Unfortunately, the origin of these statements is not given in the article.

Another recent article [20] indicates that in rabbits the pulmonary alveolar neutrophil pool accounts for only 0.19% of the total turnover of blood neutrophils. If this is true, it appears that the lung also contributes only minimally to the total capacity of the body to dispose

of neutrophils.

Even if neutrophils have not been shown to be a significant feature of the normal intestine, there are many reports of neutrophils in the lumen of the intestine. Neutrophils are a feature of intestinal inflammations such as those due to Salmonella or Shigella. However the presence of neutrophils in the lumen in experimental [5] or clinical cases [43] of noninvasive enteric Escherichia coli might be attracted by a simpler mechanism than the full-blown inflammation elicited by invasive bacteria. A simpler system is always attractive in experimental pathology; a simple system might be easier to analyze.

Research regarding enteroluminal neutrophils and porcine colibacillosis began with the demonstration that neutrophils can be attracted into ligated intestinal loops of swine if those loops were inoculated with E. coli [107]. Enteroluminal neutrophils were later attracted into ligated intestinal loops of immune swine if the soluble antigen was placed in the lumina of the loops [8]. The fact that this work has not been repeated will be discussed briefly below. The potential protective effects of enteroluminal neutrophils were addressed indirectly by examining the proliferation of encapsulated and nonencapsulated, piliated and nonpiliated strains of enterotoxigenic E. coli in intestinal loops [34]. Experiments using Thiry-Vella loops in swine supported the idea that enteroluminal neutrophils could play an important protective role [9,10].

The experiments mentioned in the preceding paragraph will be

described more completely below in a roughly chronological order.

E. coli in ligated intestinal loops of swine can attract neutrophils into the intestinal lumen

This line of experimentation was initiated by Yong [107] with 6-kg pigs and two different strains of E. coli. One enteropathogenic strain (serotype O138 K81:NM) and one nonenteropathogenic strain, E. coli strain 123 were used. According to the way the term "enteropathogenic" was used in literature at that time, it probably referred to a strain which not only elaborated an enterotoxin but which was also capable of colonizing the intestine. Ligated intestinal loops were created and were inoculated with the two different strains of E. coli. Both strains stimulated the emigration of neutrophils into the intestinal lumen. Inocula other than the bacterial strains were tried in the loops: bacterial extracts (the extract of the enterotoxigenic E. coli contained enterotoxin, although the type of enterotoxin was not specified), polyethylene glycol, and a detergent. None of these resulted in appreciable attraction of neutrophils into the lumen. The enterotoxin preparation caused fluid accumulation as early as 1 hour.

Occasionally, enteroluminal neutrophils had phagosomes with bacteria. This was particularly noted if loops also contained serum, especially immune serum.

Counting enteroluminal cells, which is crucial to evaluating the neutrophil response, has been a problem in the past. Yong used a hemocytometer. Luminal cells are not distributed evenly in fluid as are

most blood cells so the hemocytometer does not give accurate estimates of cell concentrations. Any statements based on quantification of enteroluminal cells using a hemocytometer must be taken "with a grain of salt."

Given that disclaimer, Yong found no difference between enteropathogenic and nonenteropathogenic E. coli in ability to elicit neutrophils. Histological changes of loops inoculated with E. coli 123 were milder than those inoculated with the pathogenic strain (E. coli 0138). The anterior small intestine yielded more enteroluminal neutrophils than did the posterior small intestine.

Immune-mediated emigration of neutrophils into porcine small intestine

A series of experiments performed by Bellamy and Nielsen [3,4,7,8] using ligated intestinal loops in swine demonstrated that neutrophils could be attracted into the lumen of the intestine if a recognized antigen was in that lumen. Briefly, pigs were immunized to bovine serum albumin (BSA). Subsequent placement of BSA into ligated intestinal loops attracted neutrophils into the lumen of the intestine.

Total cells within the intestinal lumen were counted by use of an automatic cell counter. Luminal contents were first sonicated to disrupt cell aggregates without affecting total cell count. Although this method is different from that of Yong, it is still not ideal and again the data based on total numbers of cells within the lumen of the intestine should interpreted in light of that fact.

Numbers of neutrophils attracted by this immune-mediated mechanism

were comparable to the number attracted by an enterotoxigenic strain of E. coli. After non-immune pigs received immune serum intravenously, their neutrophils migrated into the lumina of intestinal ligated loops when BSA was in the loops. The neutrophil response in these passively-immunized pigs was approximately one-fifth of the response seen in actively immunized pigs. Attempts to passively transfer immunity either by lymphocytes from immune pigs or by a conditioned medium (immune lymphocytes were exposed to antigen) were inconclusive.

Although the point was not emphasized, an examination of the data [3] reveals that although neutrophils will move into the intestinal lumen in response to the luminal presence of bacteria, the additional presence of a recognized antigen boosts the neutrophilic response nearly seven-fold.

Subsequent work [4] showed that parenteral immunization of pigs with E. coli increased the number of E. coli phagocytosed by enteroluminal neutrophils. This immunization resulted in a reduction of enteroluminal E. coli of nearly 100-fold. Bellamy and Nielsen concluded that the reduction of luminal bacteria was due to effects of the neutrophils, whether through phagocytosis or other mechanisms. Still another investigation [6] demonstrated that fluid and electrolyte movements in the intestine were not significantly altered during immune-mediated enteroluminal neutrophil emigration.

The attraction of PMNs into ligated intestinal loops containing BSA in BSA-immune pigs was compared to the Arthus reaction elicited by intracutaneous inoculation of BSA in these same pigs [7]. Immune-mediated

enteroluminal neutrophil emigration was characterized as protective. No hemorrhage, edema, thrombosis, or signs of excessive fluid in loops occurred as a result of immune-mediated neutrophil emigration. In the skin of immune animals inoculated subcutaneously with the antigen BSA edema, hemorrhage, thrombosis, and accumulation of PMNs were noticed as is typical of an Arthus reaction. Bellamy and Nielsen proposed that the intestine is more refractory to edema (because of its abundant blood and lymph supply) and also that in the intestine the antigen and PMN's are able to pass on down the GI tract while in a cutaneous Arthus reaction, the antigen and exudate persist.

Capsule contributes to persistence of enteric enterotoxigenic E. coli

Virulence factors of enteropathogenic E. coli which might be important in colonization and/or persistance of the bacterium in the porcine small intestine were addressed by Hadad and Gyles [34,35]. As was mentioned before, the term "enteropathogenic" at that time was taken to mean E. coli that not only elaborated enterotoxin but which were also capable of colonizing the intestine.

Calves were orally infected with the enterotoxigenic E. coli [35] and were euthanized at 16 hours post-infection (PI). Villi in the middle and distal small intestine were infiltrated with PMNs. Tissues were examined by transmission electron microscopy; the bacterial capsule was defined by staining with ruthenium red. Capsular material was close enough to the microvilli to be mediating adhesion, and was produced in abundant amounts.

In a second set of experiments [34], pairs of ligated loops were created in the small intestine of calves. Both loops contained E. coli 505 NalC (O101:K28,K99). One loop in each pair also contained 1 ml of antiserum to either the O antigen or to both the O and K antigens (O101:K28). After 14 hours of incubation, fluid in the loops was measured and E. coli viable counts were determined. If anti-K antibody was present, then fluid accumulation in the loops was greatly diminished. The hypothesis was advanced that perhaps the capsule enhanced virulence by inhibiting phagocytosis. When homologous anti-K or anti-OK antiserum was used, reduction of viable E. coli counts was more than 99%.

This work demonstrated that neutrophils were a feature of the cellular infiltrate, and that capsule significantly contributed to persistance of noninvasive pathogenic intestinal E. coli.

One weakness of this work is that antiserum is known to be present in the system; agglutination of bacteria may be the cause of the reduction in colony counts.

Enteroluminal neutrophils in porcine Thiry-Vella loops inoculated with E. coli

Isolated intestinal segments were created in swine duodena; these segments communicated at both ends with the exterior of the animal. These Thiry-Vella loops were inoculated with a particular strain of E. coli and flushed. Subsequently, they were re-inoculated with the same strain many times. A rapid decline of the E. coli was noted in the "immune" loops (loops which had been exposed to the E. coli before) and

this decline was associated with the rapid and marked influx of neutrophils into the Thiry-Vella loop [9,10].

This body of work is difficult to interpret and even more difficult to summarize. The experimental design was never clearly stated. Crucial techniques were not described. For example, the clearing of the Thiry-Vella loops of the E. coli is crucial, yet the method for determining the number of E. coli in the loops was not stated.

The methods used to assess neutrophil numbers were no more accurate than those described previously. In this work, lactoferrin and lysozyme were used as markers of neutrophils; lactoferrin and lysozyme were quantified. Numbers of neutrophils were determined although the method by which neutrophils were quantified was not stated. There is no assurance that lactoferrin and lysozyme in the intestinal lumen could only have come from neutrophils.

Addition of iron to the loops reduced the killing of the E. coli. This was interpreted to indicate that lactoferrin from neutrophils was a primary bactericidal mechanism. However, it has been documented that iron will interfere with oxygen-metabolite mediated bactericidal actions of rabbit neutrophils, perhaps by decreasing H_2O_2 [28,45].

This work suffers from too many inadequacies and cannot stand alone. However it does lend support to the experiments mentioned previously in this review.

Immune colostrum and enteroluminal neutrophils in neonatal pigs

Genetic susceptibility or resistance of individual pigs to enteric colonization by E. coli bearing the pilus antigen K88 is inherited in a Mendelian fashion as an autosomal recessive trait. Animals which do not have the receptor are resistant to colonization by the K88-positive E. coli and do not develop a strong immune response to it. Animals which do have the receptor are susceptible to colonization by the K88-positive E. coli and after such colonization show antibodies to K88. It is possible for susceptible piglets to be born to resistant sows. The colostrum of the resistant sows is much less protective against K88-positive E. coli than is the colostrum of susceptible sows [84].

Because of access to a special swine herd in which the resistance or susceptibility of each pig to colonization by the K88-positive E. coli is known, Sellwood [84] was able to measure in vitro attributes of colostrum from various sows and to compare them with actual clinical protection or lack of protection provided to piglets.

Initially, the different attributes of various samples of colostrum were measured in vitro. The colostral attributes which correlated with clinical protection were (1) prevention of adhesion of K88-positive E. coli to brush-border isolates and (2) promotion of phagocytosis by neutrophils presumably through opsonization. Fractionation of colostrum indicated that IgM and IgA fractions were most responsible for opsonization [84]. The results of this study prompted interest as to whether or not neutrophils were actually in the intestinal lumina of

neonatal pigs.

A different series of experiments was begun to try to define under what circumstances neutrophils might be in the intestinal lumina of neonatal piglets. Preliminary results [37] and a more complete report [85] have been published. The genetically-defined swine herd was again used (genetically-defined in terms of the K88 receptor, at least). The data presented do not totally support the conclusions. An excerpt from the publication reads:

> It was concluded that neutrophil emigration into the intestinal lumen of piglets could occur in response to K88-positive E. coli and resulted not from the presence or absence of the intestinal K88-receptor but from the ingestion of immune colostrum.

However, within the paper is a table showing that susceptible piglets could suckle susceptible sows (thereby receiving immune colostrum) or resistant sows (thereby receiving nonimmune colostrum) and that these two groups of piglets would both respond with neutrophils in the intestinal lumen. It would appear that the presence of immune colostrum in the intestinal lumen is not the only factor which determines whether or not neutrophils will move into the lumen in response to K88-positive bacteria.

This work also suffers from a problem in counting total cells in the intestinal lumen. Sellwood used a counting chamber to determine total cell counts of luminal fluids. Personal experience indicates that method

is not accurate for luminal fluids. A scanning electron micrograph within this paper [85] shows a mat of cells which would be unlikely to disperse evenly for accurate counting in a chamber.

Summary of enteroluminal neutrophils in porcine colibacillosis

Five different reports of research have been reviewed. The three strongest reports are the first three described in this review. It has been clearly demonstrated that noninvasive E. coli within the porcine intestinal lumen can attract neutrophils into the lumen. Bellamy demonstrated that the attraction of neutrophils into the porcine intestinal lumen was in large part due to immunity to the luminal antigen. Unfortunately, Bellamy's work has not been able to be repeated (R. Rose, NADC, Ames, Iowa, unpublished data; J. Keirby, Dept. of Animal Husbandry, University of Bristol, Bristol, England, personal communication, Nov. 11, 1982). It may be that Bellamy by chance struck just the right combination of genetics and immune history in his experimental pigs. Tolerance to particular antigens is a well-known feature of intestinal mucosal immunity; perhaps researchers trying to repeat this work have been troubled by immune tolerance in the pigs. Although the reports of the immune-mediated emigration of neutrophils into the intestine appear to be so complete and well-described, apparently some crucial factor necessary for replication is as yet unidentified.

The demonstration that presence of capsule enhances colonization of the intestine by noninvasive E. coli is intriguing; one can consider that

perhaps capsule serves an anti-phagocytic function in the intestinal lumen just as it does in the bloodstream. This possibility has not been investigated.

The ability of enteroluminal neutrophils to function in the intestinal lumen is suggested by the fall in bacterial counts associated with enteroluminal neutrophils [4]. A more complete description of the functional capacity of enteroluminal neutrophils awaits further work. Cytochemical stains are attractive in that they probe the enteroluminal neutrophils *in situ*; *in vitro* assays might also yield valuable information.

Neutrophils in other parts of the gastrointestinal tract

Neutrophils are found consistently in the oral cavity, specifically in the gingival crevice. Because this site is easily accessible to investigators, work continues to be done on neutrophils in the gingival crevice. Neutrophils in other parts of the gastrointestinal tract have not been investigated to any significant degree.

Somewhat circumstantial evidence that neutrophils play an important role in normal oral health is provided by Charon, Mergenhagen, and Gallin [17]. Using dentist's indices for plaque, gingival inflammation, and gingivitis, these investigators evaluated the oral mucosa and teeth of patients with neutrophil dysfunction and of properly matched controls. Patients with neutrophil dysfunction, regardless of age, were more susceptible to gingival disease and to ulceration of the oral mucosa. Three out of the twenty-seven patients examined were even edentulous.

The effect of topical antigen on the gingiva of sensitized rabbits has been examined [56], and the tissue appearance has been compared to a typical Arthus reaction elicited elsewhere on the bodies of the same rabbits. The antigen used was horseradish peroxidase which can be detected cytochemically. In nonsensitized rabbits, the horseradish peroxidase was distributed evenly throughout the intercellular spaces of the epithelium. In the sensitized rabbits, the horseradish peroxidase occurred as large discrete clumps in intercellular spaces of the epithelium. There was no evidence of an Arthus reaction in the gingiva although peroxidase injected elsewhere in sensitized rabbits elicited a typical Arthus reaction characterized by platelet-fibrin thrombi and a neutrophilic vasculitis. The author speculated that the immune response in the gingiva was successful in preventing access of the antigen to connective and vascular tissues. He did note that neutrophils migrated into the region of antigen-antibody precipitate. This parallels Bellamy's observation of a "protective" neutrophilic response to luminal antigen in an immune animal [7].

Brownstein and Johnson [12] induced leukopenia in mice through the use of cyclophosphamide. They applied Pseudomonas aeruginosa to the nasal mucosa of both leukopenic and normal mice. (Bacteremia in mice can be initiated when P. aeruginosa penetrates nasal and oral mucous membranes.) P. aeruginosa penetrates apparently intact conjunctival and nasal mucosa of agranulocytic rabbits and mice, respectively, but is unable to do this in normal animals. In the experimental mice which were not neutropenic,

the applied P. aeruginosa attracted many neutrophils which traversed otherwise unaltered epithelium. Neutrophils on the mucosal surface contained cytoplasmic bacilli which stained for Pseudomonas antigens. The neutrophils cleared the nasal mucosa of these organisms by 18 hours. In the leukopenic mice, the P. aeruginosa was able to penetrate the nasal epithelium. Undermined epithelium sloughed in sheets creating large areas of denuded mucosa.

Enteric Escherichia coli Infection

In this part of the review, clinical syndromes will be discussed first; toxins will be addressed separately. This is an artificial division, as one cannot speak of clinical syndromes without speaking of the toxins involved. In return, the effects of the toxins are understood through examination of the clinical syndromes. However artificial it might be, however, the two subjects will be dealt with separately as much as possible. It is hoped that this will promote insight rather than confusion.

Clinical syndromes

In the following discussion, clinical syndromes are grouped according to type. Specific examples are given to illustrate the different syndromes.

Explanation of terminology: ETEC, EIEC, EPEC, EHEC Although different investigators occasionally use a term in an unusual manner, there is consensus on the nomenclature of enteric colibacillosis.

Because terminology evolved over time, some older papers may refer to an enterotoxigenic strain as being enteropathogenic [73]. At the time these papers were written, the thinking was that a strain of E. coli might produce an enterotoxin but it could not be enteropathogenic (i.e. produce intestinal disease) unless it also had an adhesin to promote colonization of the intestine [59].

When new pathogenic mechanisms are being investigated, researchers rely heavily on E. coli strains of certain serotypes, and one will find publications written about particular serotypes.

There are four well established categories of pathogenic E. coli which infect the intestine: enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), enteropathogenic E. coli (EPEC), and enterohemorrhagic E. coli (EHEC).

Some authors define EPEC as being strains of E. coli which cause intestinal disease but which are not enterotoxigenic or enteroinvasive [95]. This definition, while accurate, is not a very complete one. A perspective which better serves the reader is presented by two other authors [11,31]. Historically, epidemiological studies characterizing outbreaks of epidemic infantile enteritis revealed that seventeen O serogroups of E. coli were associated with this disease.

> While ETEC and EIEC are defined on the basis of known pathogenic mechanisms, EPEC are defined by serotype. It is not surprising therefore that new knowledge suggests that the EPEC group may

include strains with a variety of potentially pathogenic characters [31].

Enterotoxigenic E. coli (ETEC) While the EPEC were originally defined by serotype, the ETEC are defined by their ability to cause a secretory diarrhea through the action of enterotoxins. The extent of the secretory diarrhea can be such that affected hosts die from dehydration and acidosis [61]. ETEC infections are common in calves and piglets during the first week of life; such infections can also be found in lambs. The ETEC are common causes of traveler's diarrhea in people [31] and are a major cause of childhood diarrhea in developing countries [11]. Necropsy of an affected calf occasionally reveals cattarhal, suppurative, or hemorrhagic enteritis, but often intestinal tracts appear completely normal. Intestines of affected pigs usually have no sign of inflammation; sometimes the presence of fluid contents in the spiral colon is the only unusual finding [2]. The ETEC are for the most part serologically unrelated to the EPEC [27].

Enteroinvasive E. coli (EIEC) The EIEC cause illness by invading the intestinal mucosa. The first report of diarrhea due to EIEC in people was in 1943; signs included pyrexia and diarrhea, with blood and mucus in stools. Stools were frequent and small in volume. Serotypes associated with EIEC are O124, O136, and O143. They resemble the shigellae in many respects; there is invasion of epithelial cells followed by intracellular multiplication. Interestingly, most of the serogroups to which the EIEC belong are antigenically related to

serogroups of shigella [31].

Enteropathogenic E. coli (EPEC) EPEC infections in human infants are not the problem they once were in industrialized countries. However these infections in human infants are still important in tropical countries [31]. The pathogenic mechanisms of EPEC are not totally clear. Strains of E. coli contained within this group may exhibit a variety of pathogenic patterns. For example, some strains of E. coli belonging to EPEC serogroups O114 and O128 produce a heat-stable enterotoxin (ST) and/or a heat-labile enterotoxin (LT), and perhaps these particular strains should be reclassified as ETEC. One of the pathogenic mechanisms of EPEC might be the elaboration of one or more cytotoxins which resemble Shiga toxin elaborated by Shigella dysenteriae [31].

Several strains of EPEC have been demonstrated to be AEEC, or attaching and effacing E. coli. The term "attaching and effacing" refers to an intimate association between the E. coli and the epithelial cell and to the effacement of microvilli in the vicinity of the particular bacterium. When infected tissues are examined by transmission electron microscopy, the E. coli are seen to be intimately attached to epithelial cells. The space between each bacterium and the epithelial cell is considerably less than the space seen with the ETEC in calves and pigs. In fact, the bacteria and the epithelial cells appear to be in direct apposition. The epithelial cell forms a pedestal for each bacterium; the pedestal in turn forms a slight cup where it adjoins the bacterium. Just below this cupping, in the cytoplasm of the epithelial cell, there are

electron-dense modifications of the terminal web area. The microvilli are effaced. Moon et al. stated "it may be that most strains currently designated as EPEC are AEEC" [62]. A description of EPEC in a human infant pre-dates the AEEC terminology, but the electron micrographs demonstrate the same pedestal formation, cupping, etc. [96]. The author states that histologic and ultrastructural changes are identical to those reported by Cantey and Blake in their description of diarrhea in rabbits [14]. Several descriptions of E. coli intestinal infections in rabbits indicate that AEEC are common in rabbits, both in infant and in weaned rabbits [14,15,75,91]. In fact, it is probable that all cases of E. coli enteritis in rabbits are due to these EPEC [71].

Enterohemorrhagic E. coli (EHEC) Several strains of E. coli, notably serotype O157:H7, have been associated with hemorrhagic colitis in humans. New terminology has been introduced to describe E. coli that produce hemorrhagic colitis. It is suggested strains of this type should be called entero-hemorrhagic E. coli, or EHEC [31]. A group of British researchers have experimentally reproduced hemorrhagic colitis in calves with E. coli O5:K-:H- [16,36]. Affected calves were three weeks of age or younger. Transmission electron micrographs of this infection in calves demonstrated a lesion morphologically identical to that of the AEEC: bacteria are intimately apposed to epithelial cells, there is effacement of the microvilli, and the epithelial cell cytoplasm forms pedestals with cupping. An adhesin probably is involved but it is not the pilus K99 [36]. Another isolate of E. coli O5:K4:H- was found in a 2-day-old calf

presented with mild diarrhea. When this strain was inoculated into lambs and calves, some animals responded with diarrhea, and some of those animals had blood in the stool. The colon was colonized with AEEC [63]. Some E. coli are known to produce a cytotoxin which can act on a line of cultured cells called Vero cells. These E. coli can be referred to as VTEC (Vero toxic E. coli). The categories of EHEC, VTEC, and AEEC overlap [71].

Post-weaning clinical syndromes in pigs There are four clinical syndromes in recently-weaned pigs caused by noninvasive intestinal E. coli: shock or sudden death, hemorrhagic enteritis, uncomplicated post-weaning diarrhea, and edema disease. These four clinical syndromes are not as well understood as are the ETEC infections in neonates. Two authors categorized these clinical syndromes as ETEC infections [95,105]. Some of the strains causing post-weaning diarrhea and edema disease are classified as ETEC because they elaborate one of the heat-stable enterotoxins. However, they also produce a cytotoxin [71].

There is some overlap of these four clinical syndromes. Usually only one syndrome is noted in an individual animal, but two or more may appear in a swine herd simultaneously. Jones and Smith infected weaned pigs with E. coli O141:K85ac by oral inoculation. Diarrhea was observed in some pigs while other pigs suffered from edema disease [42].

These four clinical syndromes are thought to be precipitated by the change of diet which occurs at weaning and perhaps also by the loss of passive immunity furnished in sows' milk. The porcine small intestine

absorbs water inefficiently in the post-weaning period; this may sensitize the pig to the effect of E. coli enterotoxins [29]. The experience of weaning apparently allows the proliferation of enteropathogens in the small intestine, notably E. coli.

E. coli serotype O149:K88 is most commonly associated with post-weaning diarrhea [95]. The three serotypes described in association with edema disease are also found in association with post-weaning diarrhea (O138, O139, and O141) as well as serotypes O149:K91, O8, and O157 [38,44,90]. The strains of E. coli which have been associated with these field cases have been hemolytic, but any role of a hemolysin is not understood [38].

Post-weaning diarrhea The common clinical signs are pyrexia, diarrhea, dehydration, and emaciation. Affected pigs may have neurological disturbances similar to those of edema disease, i.e., ataxia, blindness, and convulsions. Sometimes the sudden death of one or two animals is the first sign of the disease in a herd. While edema disease affects only a few animals in a herd, post-weaning diarrhea (PWD) affects most of the pigs in a herd, and most of those pigs recover. Diarrhea is presumed to be mediated by an enterotoxin [44]. In addition to the hypersecretion induced by ETEC enterotoxins (which is characteristic of infection in neonates), PWD is presumably further amplified by brush border malfunction and by malabsorption resulting from mucosal damage associated with the sudden change in diet at weaning [95].

Living animals are characterized by a yellowish diarrhea staining the

perineum [44]. Gastric infarction is the most consistently noted lesion. In one study, the posterior jejunum of nearly two-thirds of the pigs experiencing post-weaning diarrhea contained blood-stained liquid; the affected intestinal mucosa had massive, diffuse petechial hemorrhages [90]. An earlier report also indicated that microscopic hemorrhage was a common finding in the small intestine. In addition to infarction of the gastric fundus, granular thrombi can be found in mucosal veins and capillaries [2]. Jubb, Kennedy, and Palmer stated that in fatal cases there are bluish discoloration of the skin and evidence of dehydration. The small intestine may be normal in color with creamy contents, or the mucosa of the distal small intestine will be congested and the contents watery or perhaps blood-stained [44].

Shock The shock syndrome in newly weaned pigs is characterized by the sudden death of one or two piglets in a litter. This syndrome can be part of the post-weaning diarrhea syndrome. Upon necropsy, edema is noted in the intestine, lungs, kidneys, and CNS. Serous exudate is found in body cavities. These phenomena may be due to the rapid absorption of endotoxin from the intestine [44,105]. This type of fatal shock, along with edema disease, comprise "enterotoxemic colibacillosis" [65].

Hemorrhagic enteritis Hemorrhagic enteritis associated with *E. coli* is also characterized by sudden death in newly weaned pigs. Upon necropsy, hemorrhagic lesions are noted in the intestinal mucosa and in associated lymph nodes. Diarrhea may or may not occur. A hypothesis is

that this syndrome may also be due to the rapid absorption of endotoxin from the intestine [105]. Hemorrhagic colitis in humans has been associated with E. coli strain O157:H7 which has been shown to secrete a cytotoxin [41,77].

Edema disease Edema disease is an acute disease of young pigs which typically occurs about one week after weaning [105], although it has been reported in pigs only four days old [44]. Edema disease is associated with enteric colonization by certain serotypes of E. coli, most of which are hemolytic. The disease often occurs in association with outbreaks of post-weaning diarrhea [44]. Diarrhea and edema disease do not usually occur in the same animal [42,44]. Only a few serotypes are involved in the disease: O138:K81, O139:K82, and O141:K85. The O138 and O141 strains also produce enterotoxin, and so a secretory diarrhea may be part of the clinical picture if those strains are the infecting agents. These strains colonize the small intestine during the disease [65]. No adhesin has been identified [57]. The cardinal signs of edema disease are neurological disorders (ataxia, convulsions, blindness, paralysis) accompanied by edema of the subcutis in the forehead and eyelids [22,44]. When a necropsy is performed, straw-colored or sanguineous edema is found throughout the carcass, especially at the cardiac region of the stomach, the mesentery, between the spiral coils of the spiral colon, the eyelids, over the frontal bones, the lungs, and occasionally along the ventral midline [2]. Although most evidence supports the idea that edema disease is due to the effects of a single toxin, edema disease principle, some

people propose that edema disease is due to the effects of endotoxin or due to a hypersensitivity to specific E. coli antigens [65]. However, the vascular lesions are not typical of either endotoxic shock or of a systemic hypersensitivity.

Edema disease is a classical example of enterotoxemia. The toxin is absorbed from the intestine but is active at another site. Gross or microscopic lesions other than vascular lesions and edema are not found in the intestine [57]. Small arteries and arterioles throughout the body are affected. Early vascular changes include swelling of endothelial cells and pykonosis and karyorrhexis of smooth muscle nuclei, often accompanied by fibrinoid degeneration or hyaline change. In more advanced lesions, proliferative mesenchymal elements are found in the tunica media and tunica adventitia. Neither inflammation nor thrombosis is a classical feature of the angiopathy [44].

A comparison has been drawn between edema disease and the clinical syndrome of hemolytic uremic syndrome (HUS) which is occasionally seen in people following hemorrhagic colitis [13]. However, hemolytic uremic syndrome in people is usually characterized by numerous fibrin thrombi with microangiopathic hemolytic anemia. Disseminated intravascular coagulation is thought to be due to endotoxin [78]. Although this is not similar to the usual vascular lesions of edema disease, a role of endotoxin in edema disease has not been excluded [57].

Toxins of enteric Escherichia coli

Cytotonic enterotoxins are those toxins (e.g., cholera toxin) which induce net fluid secretion by interfering with biochemical regulatory mechanisms without causing overt histological damage. A cytotoxic enterotoxin is a toxin which induces actual damage to intestinal cells as a necessary prelude to onset of net fluid secretion [89]. In this discussion, an enterotoxin is a bacterial toxin which acts directly on intestinal tissues and which, unless specified otherwise, has a cytotonic effect.

Enterotoxins The enterotoxins are divided into two main groups according to their stability upon heating. There is one well described heat-labile enterotoxin; there are two well described heat-stable enterotoxins.

Heat-labile enterotoxin (LT) Heat-labile enterotoxin (LT) consists of two polypeptide subunits. Subunit A has a molecular weight of about 25,000 and has the ability to stimulate adenylate cyclase activity. Subunit B has a molecular weight of about 11,500 and forms aggregates of five monomers which are able to adsorb to one of the many types of sensitive cells, e.g., the Y1 adrenal cells used in in vitro tests for LT activity. Immunological neutralization tests show that there is a close similarity between E. coli LT and the Vibrio cholerae enterotoxin, or cholera toxin (CT). Furthermore, there is significant homology between the amino acid sequences of the B subunits of LT and CT [31]. In both LT and CT the B subunit is responsible for binding to the

Gm 1 ganglioside of the epithelial cells of the small intestine, while subunit A stimulates adenylate cyclase activity, thereby increasing the concentration of cyclic AMP in the cells. It is not clear exactly how fluid secretion is mediated [89]. The increased level of cAMP may act in two ways to cause fluid and electrolyte loss into the intestinal lumen. Field [25,76] has postulated that LT causes an increase in cAMP in both villous absorptive cells and crypt secretory cells. This increase in cAMP then leads to both an antiabsorptive action on villous cells and a direct secretory action on crypt cells. The antiabsorptive action on villous cells might be due to cAMP inhibiting cotransport of NaCl across the brush border into the cell. In the crypt cells, increased cAMP might allow Cl^- ions to pass out of the cell through the luminal membrane.

Shiga toxin, which will be discussed below, also has a B subunit which binds to the surface of mammalian cells. Shiga toxin also has five B subunits per holotoxin. Although there is no antigenic cross-reactivity between Shiga toxin and LT or CT, amino acid sequence data indicates that the B subunits of E. coli heat-labile toxin and cholera toxin are distantly related to the B subunit of Shiga toxin [66].

It is thought that crypt cells are responsible for most of the fluid accumulation in the intestinal lumen. Certainly another secretagogue which acts through cAMP, prostaglandin E_1, acts predominantly through crypt cells. Destruction of villous cells in porcine small intestine through the action of transmissible gastroenteritis virus (TGE) does not affect the secretory response of small intestine to prostaglandin E_1 [102].

At one time, it was doubted that the crypt and villus cells could reliably be said to respond differently to enterotoxins [27]. That concept has waned because of a new understanding that enzyme and nucleotide concentrations can differ in these two populations of intestinal epithelial cells [32,76].

Tissue culture methods using Y1 mouse adrenal cells [23] and Chinese hamster ovary cells [33] are the standard tests for the detection of LT, although other cell lines, including Vero monkey kidney cells, are also sensitive. In these tests the tissue culture cells are exposed to bacterial culture supernatants, and when LT or cholera toxin is present the intracellular concentration of cAMP increases, leading to a morphological response which can be seen microscopically. Chinese hamster ovary cells elongate, while Y1 cells become rounded. The cytotonic response is distinct from the cytotoxic effect of supernatants of some E. coli O26, O126, and O157 cultures on Vero cells [31,89]. The result of LT is secretion into the lumen of the proximal small intestine of electrolyte-rich fluid which will appear clinically as watery diarrhea [95].

There are two different LTs. Antibody to classical heat-labile enterotoxin (LT) of strain H10407 did not neutralize LT of strain HSC10, but both enterotoxins induced similar effects on Y1 and Chinese hamster ovary cells and rabbit ileal loops [55]. The two different LT's have been purified [39].

Heat-stable enterotoxins Two types of heat-stable

enterotoxins can be produced by enterotoxigenic E. coli. They can be differentiated by many criteria: biological, biochemical, and physiological [89].

Heat-stable enterotoxin a (STa) is produced by strains of human and animal origin, while heat-stable enterotoxin b (STb) is produced by strains that are usually of porcine origin [31].

Some authors still use the notation ST when they are speaking of STa [32]. Because they are working with strains of E. coli which are enterotoxigenic in people and because they refer to cGMP-mediated secretion, they are apparently speaking of STa.

Heat-stable enterotoxin a (STa) is methanol-soluble. It is active in infant mice, neonatal pigs, rabbit ileal loops, and rat intestinal loops [89]. The nomenclature of the STa group of toxins can be confusing. STa also is known as ST_A or ST_I. Studies using genetic probes have shown that STa can be further divided into two types, ST_{A1} and ST_{A2}. ST_{A1} has been found in strains from human and animal sources while ST_{A2} has been found only in human strains. ST_{A1} is also known as ST_{Ia} or ST_p (the "P" stands for porcine). ST_{A2} can be known as ST_{Ib} or ST_h (the "h" stands for human) [31].

STa does not affect the concentration of cAMP but instead stimulates the activity of guanylate cyclase, causing an increase in cGMP levels. This occurs only in intestinal epithelial cells and not in a variety of other tissues and cell lines, suggesting that a unique toxin receptor is present in intestinal cells [31]. The receptors for STa are distinct from

those for LT or for CT [27]. The activation of the guanylate cyclase-cGMP system results in impaired NaCl absorption and in Cl^- secretion [32]. The guanylate cyclase which is activated by STa is a particulate guanylate cyclase. This specific guanylate cyclase and the STa receptor are independent proteins coupled by cytoskeletal components. The increase in cGMP may act through a cGMP-dependent protein kinase and its protein substrate to influence channels for ion transport [98]. The intestinal secretion induced by STa is not mediated solely by crypt cells (at least in pigs); the full secretory response to STa is dependent on the integrity of the villous epithelium [102]. Guanylate cyclase activity is greatest in differentiated villus cells and least in proliferating crypt cells [32].

Some authors include STa in the group of toxins which act on or within membranes mainly because the effects of STa are rapidly and completely reversed by rinsing affected tissues [89]. This integrates well with the concept of a particulate guanylate cyclase which is tightly membrane-associated and which is activated by direct and continued interaction with a toxin-receptor complex.

The most widely used bioassay for STa is the infant mouse test. In this test, culture supernatants are injected into the stomach of infant mice. After four hours the mice are killed, and the intestines are examined for dilation due to accumulated fluid. The intestines are then removed and the ratio of intestinal weight to remaining body weight determined as an objective measure of fluid accumulation [31]. There is no

assay using cultured cells which can be used to detect STa.

Heat-stable enterotoxin b (STb) is methanol-insoluble and has been defined as being inactive in infant mice and active in ligated intestinal loops of older piglets and rabbits. Species specificity of the STb response needs to be re-examined in light of the recent finding that STb is susceptible to degradation by intestinal proteases [100]. STb is also known as ST_B or ST_{II}. The fluid secretion induced by STb appears to take place with no alteration of cGMP levels, and the mechanism of action of STb remains unknown [31]. STb may stimulate the secretion of HCO_3^- [89]. As is also the case for STa, the secretory response to STb in pigs depends on the integrity of the villous epithelium [102]. STb has been considered to be an enterotoxin (cytotonic), but recent work indicates it may be cytotoxic as well [101]. See Part II of this thesis.

Strains of E. coli which produced STb but not LT or STa have been shown to cause diarrhea in neonatal piglets [48,49,60].

Kennedy et al. [52] have found that in their hands STb is a cytotonic toxin and does not cause histologic damage in the intestines of mice, rats, rabbits, and piglets.

In contrast to the findings of Kennedy et al., Whipp et al. found that E. coli strain 1261, which produced STb as well as STa, caused partial villous atrophy in 3-week-old pigs [102]. These findings are confirmed in Part II of this thesis. Additional confirmation was acquired through further work by Whipp et al.; interestingly, partial villous atrophy associated with presence of STb in a ligated loop

occurred independently of secretion due to STb [101].

Cytotoxins The nomenclature of the E. coli cytotoxins has been confusing. Some of that confusion is waning because certain toxins have been found to be identical to others (i.e., Vero toxin 1 is equivalent to Shiga-like toxin I, and Vero toxin 2 is equivalent to Shiga-like toxin II).

Vero toxins Historically, the Vero toxins were discovered first. In 1977 and 1978, Konowalchuk et al. described the activities of five different heat-labile toxins on cultured Vero cells (which are kidney cells from African green monkeys). Two of these toxins were cytotonic and had actions typical of LT's; they were antigenically different from each other. The remaining three toxins were cytotoxic to the Vero cells; these toxins were referred to as Vero toxins. One of the toxins was produced by several of the E. coli strains tested; the prototype strain was H-30. These three Vero toxins were further characterized by isoelectric focusing point, molecular weight, and cross-neutralization by different antisera. The three Vero toxins were found to be three different toxins. The two Vero toxins which were not neutralized by antiserum against strain H-30 were not antigenic in these studies [54,55]. The two nonantigenic toxins were the VT of porcine strain E57 and the VT of human strain H.I.8 [22]. All the Vero toxins were heat-labile [71].

Shiga-like toxins In 1982, the first substantial report was published indicating that some E. coli produced a toxin similar to the

Shiga toxin produced by Shigella dyseteriae 1 [67]. The E. coli toxin and the Shiga toxin appeared to be identical in agar gel immunodiffusion precipitation tests using anti-Shiga toxin as the precipitating antibody. The E. coli lysates showed the same three effects in bioassays used to measure Shiga toxin: they were enterotoxic in rabbit ileal loops, they were paralytic and lethal for mice, and they inhibited protein synthesis in HeLa cells. The E. coli toxin was referred to as Shiga-like toxin.

In 1983, another E. coli Vero toxin was described. Edema disease principle (EDP) was found to be cytotoxic for Vero cells. The cytotoxic effect was neutralized by anti-EDP serum. EDP could be differentiated from the Vero toxin of strain H-30 because it was not neutralized by antiserum against H-30. Unlike the two non-antigenic Vero toxins described by Konowalchuk et al., EDP was a good antigen [22].

A recent review by O'Brien and Holmes [66] summarizes many recent developments in this field. Briefly, two different Shiga-like toxins can be produced by different E. coli. The EHEC produce Shiga-like toxin [68,69], and indeed some strains make both types of Shiga-like toxins. These two toxins have been named Shiga-like toxin I (SLT-I) and Shiga-like toxin II (SLT-II). The Vero toxin produced by Konowalchuk's prototypic strain H-30 is identical to SLT-I. The SLT-II is identical to a toxin called Vero toxin II (VT2). Both the SLT-II and the VT2 were found to be the "second cytotoxin" elaborated by EHEC strains of the serotype O157:H7. Some of these EHEC strains of seroypte O157:H7 produce both SLT-I and SLT-II. The SLT-I predominates in cell lysates while SLT-

II is the more active toxin in culture filtrate.

One might wonder why these Shiga-like toxins are so called. What characteristics do they have such that they are referred to as similar to Shiga toxin; what characteristics keep them from being referred to as identical to Shiga toxin? They are called Shiga-like toxins because in bioassays they have effects like those of Shiga toxin: they are enterotoxigenic in rabbit ileal loops, they are paralytic and lethal for mice, and they are cytotoxic to HeLa and Vero cells [66]. At least one of the Shiga-like toxins is itself enterotoxic, but the enterotoxic effect requires 10^4 times more toxin than the cytotoxic effects. A human EHEC serotype O157:H7 which produces high levels of Shiga-like toxin (some strains in fact produce both SLT-I and SLT-II), provokes watery diarrhea in infant rabbits [16].

These particular *E. coli* toxins continue to be called Shiga-like toxins rather than Shiga toxins because they have not yet been shown to be absolutely identical to Shiga toxin in amino acid sequence. Of course both SLT-I and SLT-II cannot both be exactly like Shiga toxin; only SLT-I is neutralized by anti-Shiga toxin. The amino acid sequence of the B subunit of one Shiga-like toxin exactly agrees with the amino acid sequence inferred from the nucleotide sequence of the B subunit of Shiga toxin [66]. Perhaps one day the A subunits of these two toxins will also be found to be identical.

Edema disease principle (EDP), may also fit into the pattern of Shiga-like toxins. Preliminary work suggests that at least one Vero

toxin from edema disease-producing strains of E. coli is related to, but not identical to, the prototype SLT-II. This Vero toxin differs from SLT-II in that it is not active against HeLa cells, while SLT-II is active. However antiserum against SLT-II neutralizes the toxic effects of this Vero toxin [66].

The differences between Shiga and Shiga-like toxicity of various shigellae other than S. dysenteriae 1 are probably only due to differences in toxin yield. The amounts of toxin produced by E. coli show the same variability among different strains. Some of them, particularly EHEC O157:H7 strains and several EPEC strains such as O26, exhibit toxin production as high as that of S. dysenteriae, which is the higher producer among Shigella spp. It has been shown that rabbit diarrhea E. coli strain 1 or RDEC-1 (serotype O15:H-) produces only low to moderate amounts of Shiga-like toxin [71]. E. coli O5:K4:H-, which can cause hemorrhagic colitis in neonatal calves experimentally, has been found to be a high producer of Shiga-like toxin [63]. It may be that many bovine strains produce Shiga-like toxins. One study indicated that 3% of diarrheic calves are infected with such strains. E. coli O111 and O26 producing high levels of Shiga-like toxin exist both in humans and cattle [71].

Keenen et al. have described villous atrophy in rabbit ileal loops due to effects of Shiga toxin from S. dysenteriae or of Shiga-like toxin from E. coli O157:H7 strain 933. Ultrastructural examination revealed individual cell death of the type referred to as apoptosis. Villus

absorptive cells which were apparently still viable contained multiple large phagolysosomes [50]. Although strain 933 can produce both SLT-I and SLT-II, the preparation used contained only SLT-I [66].

Another Vero toxin has been isolated from hemolytic E. coli belonging to serotypes O75:K-, O75:K95, and O2:K2, isolated from pigs with diarrhea. This toxin induced the formation of multinucleate cells in Vero monolayers. This toxin was suspected of being similar or identical to a cytotoxic and necrotising factor (CNF) found in hemolytic strains from children with diarrhea [30].

Karmali et al. proposed that both E. coli and S. dysenteriae 1 may lead to hemorrhagic-uremic syndrome (HUS) through production of Shiga and Shiga-like toxins. The finding of antibodies to this toxin in healthy older children and in adults may explain why HUS is usually a disease of early childhood [43,46,47]. Edema disease of pigs has been compared to HUS and a similar pathogenesis (i.e., a Shiga-like toxin) has been proposed for both [46].

It is currently thought that many EPEC and all EHEC will be found to act through Shiga-like toxins [66].

Edema-disease principle The toxin, edema disease principle (EDP), is responsible for edema disease in weaned pigs and is produced by certain E. coli strains. The clinical syndrome produced by the EDP is indistinguishable from naturally occurring disease. There are three bioassays for activity of EDP: production of neurological disturbances and edema in pigs, induction of characteristic limb paralysis in mice,

and cytotoxicity of Vero cells [22]. As was mentioned above, EDP may be related to but not identical to Shiga-like toxin II [66].

Clugston and Nielsen [19] first demonstrated that the clinical signs of edema disease were not due only to endotoxin. They compared the effect of intravenous injection of a partially purified freeze-thaw extract of E. coli (referred to as edema disease principle or EDP) and phenol/water extract from the same strain (endotoxin). The clinical responses induced by EDP and endotoxin were similar initially, but EDP produced additional delayed neurological signs of ataxia characteristic of edema disease [105]. A role of endotoxin in edema disease has not been ruled out [57].

Enterotoxins and cytotoxins of Escherichia coli: conclusion

Research into the actions and interrelationships among these toxins is at an exciting point; the puzzle is coming together. This time of comprehension which follows confusion is certainly not a phenomenon unique to the study of enterotoxins and cytotoxins of E. coli. P.D. Mier and D.W.K. Cotton could have been speaking of enterotoxins and Shiga-like cytotoxins rather than of collagen:

> As in all fields of biochemistry, the first simplistic descriptive phase is followed by a phase of intensive, multidisciplinary research which, at first, leads to a state of maximum confusion. This phase of maximum confusion in collagen chemistry was represented by the era of gelatin investigations. . . . [58]

It would be difficult to say exactly what phase of enterotoxin/cytotoxin research represents the period of maximum confusion. However it is clear that this is an era of resolution. Reasons for this clarity lie as much in techniques and purification methods as they do in the accumulation of a critical amount of knowledge. Specific in vitro assays have promoted identification of different toxic factors. The availability of relatively pure products is definitely a major factor. This is especially apparent in the case of the Shiga-like toxins; amino acid sequences and specific antisera are a boon to definition of the different cytotoxins.

An understanding of mechanisms of these toxins must certainly benefit the veterinarian in the field and the physician in the clinic. Perhaps enteric-coated capsules containing proteolytic enzymes could be given to a patient suffering from STb-induced secretory diarrhea. Perhaps a toxoid could be prepared to protect pigs from EDP. Reaching the current level of understanding has been a real intellectual achievement. Application of this understanding to clinical disease will complete the total endeavor.

Neutrophil Chemotaxis

Neutrophil chemotaxis is an active field of study for two reasons. One is that neutrophils are extremely important cells in host defense, and that problems with chemotaxis (whether inherited or acquired) underly many clinically important medical problems [26]. The other reason is that the neutrophil is an excellent cell in which to study receptor-mediated

transmembrane signalling. Neutrophils from peripheral blood are easily available for study. They can respond rapidly to the binding of a chemotaxin (ligand) to a receptor with a variety of actions: orientation and movement, a burst of oxygen consumption (respiratory burst), and degranulation [79,88].

Chemotaxis is the directional migration of cells along a chemical gradient and is a receptor-mediated process. Chemotaxis involves orientation of the cell, assumption of an asymmetric structure, adherence, spreading, and then locomotion [83].

Chemotaxis is to be differentiated from chemokinesis, which is increased random movement induced by a chemical signal [104]. Chemotaxis is directional locomotion of cells; the direction of the locomotion is determined by a chemical gradient. Neutrophils assume a physical orientation toward the chemical stimulus and move toward its source.

A discussion of neutrophil chemotaxis can quite easily evolve into a multi-faceted discussion of transmembrane ligand-response coupling at the molecular level. Topics which could be addressed include: membrane depolarization and hyperpolarization; increases in cytosolic calcium; subcellular location of increased cAMP; actin-myosin interaction along with actin-binding-protein, gelsolin, and calcium; phospholipid methylation, phospholipase activation, and arachidonic acid metabolism; activation of the hexose monophosphate shunt; the NADPH oxidase; and degranulation [79,80,83,88].

There are two approaches to measuring chemotaxis of neutrophils [103].

One is to observe individual cells by time-lapse cinematography. This method is preferred when detailed information is sought; however this technique is expensive and tedious. More commonly, "end-point" assays are used. The two types of end-point assays used most frequently are the chemotaxis chamber assay and the under-agarose migration assay. These are referred to as end-point assays because at the end of an incubation time the distribution of cells as a whole population is noted. Detailed information regarding how the cells moved to assume that distribution is not obtained.

Each type of end-point chemotaxis assay has many variations. For example, different types of filters are available for use in the chemotaxis chambers. Filters are usually composed of nitrocellulose or polycarbonate. The nitrocellulose filters are thicker (140 micrometers), and with these filters the investigator has the option of evaluating how far into the filter cells have migrated (the leading-front method). Polycarbonate filters are thinner (10 micrometers), and the number of cells which migrate through the filter is evaluated. Filters are also available with different pore sizes. The polycarbonate filters usually have straight cylindrical pores, while the nitrocellulose filters usually have circuitous pores [53]. Because the polycarbonate filters are so thin, gradient formation through them is not large. The pores are separated from one another by some distance; probably most of the cell movement takes place along the top of the filter towards gradients diffusing radially from the holes [103].

To the investigator who is trying to define which substances are chemotactic and which merely stimulate locomotion (that is, which are chemokinetic), the chemotaxis chamber assay and the under-agarose migration assay are inadequate. These assays demonstrate stimulated locomotion but that stimulated locomotion may not be chemotaxis [103]. However, if one is working with a known chemotaxin such as C5a or leukotriene B_4 (LTB_4), it is probable that stimulated locomotion is indeed due to chemotaxis.

A list of substances which have been shown to stimulate chemotaxis includes: C5a, $C5a_{des\ Arg}$, $C5a_{des\ Arg}$ with "helper factor," elastin-derived peptides, N-formyl-methionine-leucine-phenylalanine (fMLP), hydroxy-eicosatetranoic acids (HETEs) including leukotrienes, some lymphokines, crystal-induced chemotactic factor or CCF (a factor produced when neutrophils engulf monosodium urate crystals), and neutrophil chemotactic factor of anaphlaxis or NCF-A (released by immunologically challenged mast cells) [24,79,83,88]. In addition, neutrophils coated with specific cytophilic antibody will move toward the antigen which the antibody is directed against, at least <u>in vitro</u> [40]. Which of these are most important <u>in vivo</u> is not known. Probably all are important in different circumstances.

The most potent of these chemoattractants <u>in vitro</u> are C5a and leukotriene B_4. There are separate receptors for C5a and for the NH_2-terminal-formylated methionyl residues (in species whose neutrophils have receptors for fMLP), and for CCF [88]. A receptor for leukotriene B_4 has

not yet been identified.

The down- or up-regulation of receptors is an important consideration in chemotaxis assays and will be discussed in the section "Neutrophils in Exudate" of this review.

Neutrophils from certain species may not react with a particular substance which has been clearly demonstrated to be a chemotaxin for neutrophils of other species. For example, porcine neutrophils do not have receptors for fMLP [18] and do not respond to fMLP in under-agarose migration assays. Equine neutrophils have a low number of receptors for fMLP; the N-formylated peptides are able to induce degranulation and production of superoxide anions in these neutrophils but are not able to induce chemotaxis [88].

One factor that can influence the activity of neutrophils in the chemotaxis assay is the number of neutrophils which are applied to either the top of a chamber or to a well in the agarose. Cell concentration will influence chemotaxis [72,92]. The density of cells required indicates that intimate cell-cell contact may be required [92]. One author found concentration of 2.3×10^6 neutrophils was sufficient for the assay [72]. Other investigators determined that if fewer than 3×10^5 neutrophils were applied to the top of a chemotaxis chamber, the response was weak [92]. Studies involving porcine neutrophils in the under-agarose migration assay revealed that at least 5×10^5 neutrophils needed to be in an agarose well in order to produce a distinct migration pattern [86].

Two groups have worked with porcine neutrophils using the under-

agarose migration assay [18,86]. The agarose mixture used in the under-agarose migration assay may or may not contain serum. Porcine neutrophils have been compared to those from humans, rabbits, and guinea pigs. The porcine neutrophils were found to be more selective than other neutrophils in that they would respond only to complement-related chemotactic factors of human and porcine origin. Porcine neutrophils did not respond to purified bacterial chemotactic factors or to synthetic N-formylmethionyl oligopeptides. In fact, binding studies failed to demonstrate the presence of N-fMLP receptors on the porcine neutrophil [18]. The second group of researchers were unable to measure porcine neutrophil chemotaxis in a chemotaxis chamber because the neutrophils would not penetrate the filters. They also found, using the under-agarose migration assay, that porcine neutrophils would not migrate toward various bacterial culture filtrates but would move toward zymosan-activated serum [86].

In summary, neutrophil chemotaxis is a useful and convenient assay of neutrophil function. The two assays used most commonly are chemotaxis chambers and the under-agarose migration assay. Although the former has not previously been used successfully in evaluation of porcine neutrophil function, the under-agarose migration assay is a proven test of porcine neutrophils' ability to respond to a chemotaxin.

Neutrophils in Exudate

Although a Danish investigator was probably not the first person to have the thought, he expressed it well [99]. He asserted that while there

was a great deal of interest in defects of circulating neutrophils, authors were assuming that the function of the circulating neutrophil truly reflected its function after migration to the inflammatory site. However, studies on the function of neutrophils which had reached the inflammatory focus were few.

Several investigators have since pursued the question of the function of neutrophils at the inflammatory focus [51,74,87,106,109,110].

Neutrophils from the peritoneal cavity

Rabbit neutrophils have been collected from peritoneal exudate; glycogen was injected intra-peritoneally to attract the neutrophils. These peritoneal neutrophils have been found to behave quite differently depending on whether they were in tissue culture medium or in exudate fluid. Adhesiveness and locomotion of the exudate cells were strongly dependent on the medium. Chemotactic responsiveness of these neutrophils also depended on the attractant used. An "all or none" type of statement could not be made to summarize either the responsiveness or adhesiveness of exudate cells, because so much depended on their immediate environment. Exudate cells did tend to aggregate more [51].

The work of two groups deserves special mention. Zimmerli, Lew, Cohen, and Waldvogel [109] approached the question of the superoxide-generating system of exudate neutrophils. This is notable because most other groups have concentrated on chemotactic responsiveness or phagocytic capabilities of exudate neutrophils as these are relatively easy to evaluate. Zimmerli et al. saw decreased ability of exudate

neutrophils to generate superoxide if the stimulus was particulate (zymosan or casein), but not if it was a soluble stimulus (phorbol myristate acetate). The exudate neutrophils were obtained from guinea pig peritoneal cavities after injection of casein. Zimmerli et al. concluded that an initial inflammatory stimulus can lead to important alterations of one of the most important antibacterial mechanisms of granulocytes, i.e., the superoxide-generating system.

Work by Zimmerli, Seligmann, and Gallin is significant [110] not only because of the quality of the research but also because of the quality of the writing. The reader is reminded that the signal for exudation is a chemotactic stimulus. Such stimuli not only attract cells but also modify the characteristics of neutrophils. Different aspects of neutrophil function (chemotaxis, superoxide generation) are not always altered in the same way by a specific chemotaxin. Further, various chemotaxins may affect a specific neutrophil function, such as responsiveness to a certain chemotaxin, in different directions. In some situations the neutrophil response is "deactivated" (diminished without recovery) by a chemotaxin. In other situations the change in neutrophil response is rapidly reversible; this is referred to as adaptation. Under still other conditions, neutrophils can be primed so that they respond in an amplified manner to a second stimulus.

Zimmerli, Seligmann, and Gallin [110] obtained their exudate neutrophils from guinea pig peritoneal cavities, into which casein or glycogen had been injected, and from human skin chambers. Comparing

exudate neutrophils with peripheral blood neutrophils of both guinea pigs and humans, they found that exudate neutrophils from both guinea pigs and humans contained about 28% less of the specific-granule marker, vitamin B12-binding protein, than did the peripheral blood neutrophils. The interpretation was that some degranulation of specific granules had occurred. The azurophil granule marker, beta-glucuronidase, was similar in exudate and peripheral blood neutrophils. Both guinea pig and human exudate neutrophils had more fMLP receptors and C3bi receptors than did peripheral blood neutrophils from the respective species. Zimmerli et al. concluded that exudation primed neutrophils for their subsequent responsiveness to fMLP, a modification that might be crucial for efficient antimicrobial host defense. The possible ramifications of increased receptors for C3bi were not discussed.

Neutrophils of burn patients

Most of the work with exudate neutrophils has been done with neutrophils which are either present in abscesses or which have been elicited into body cavities through injection of some inflammatory substance or chemotactic substance. Yamada, Hefter, Burke, and Gelfand [106] had an interest in a more diffuse population of altered neutrophils; they wondered why so many burn patients become bacteremic. Zimmerli et al. [110] also mentioned the altered function of neutrophils after thermal injury. Yamada et al. cultured fibroblasts and exposed them to heat so that neutrophils could be exposed *in vitro* to cells altered by thermal injury. Neutrophils so exposed were attracted to the injured cells,

probably by complement that had been fixed to the fibroblasts' cell surfaces through the alternate pathway. The neutrophils were then less able to respond to a chemotactic stimulus (zymosan-activated serum) and showed diminished bactericidal capacity. It also appeared that many neutrophils were physically bound to the dead tissue cells and were immobilized.

Neutrophils, cytophilic antibody, and exudate ("armed neutrophils")

Smith, Colditz, and Watson [87] attracted neutrophils into the mammary gland. Sheep were sensitized to Staphylococcus aureus. S. aureus was then injected into one side of the mammary gland. Bacillus cereus which was the heterologous antigen, was injected into the other side of the gland. The purpose of the experiment was to see if antibody-coated neutrophils were attracted preferentially to the side of the gland with the homologous antigen, S. aureus.

The work of Smith et al. is pertinent to the work of Bellamy, who immunized pigs to bovine serum albumin (BSA) and placed BSA into the lumen of the small intestine [8]. Although Bellamy did not address the question of "armed neutrophils" (neutrophils coated with cytophilic antibody against the pertinent antigen), it is a germane subject. Smith et al. designed their experiment specifically to answer questions about armed neutrophils and their presence (in greater numbers? to greater effectiveness?) in the mammary gland.

Smith et al. detected the presence of cytophilic antibody by the use of FITC-protein A followed by examination of cells under a fluorescence

microscope. The specificity of the cytophilic antibody was presumably to the protein A of Staph. aureus. The effectiveness of the cytophilic antibody in promoting phagocytosis of the homologous antigen was arrived at through a series of phagocytosis and opsonization protocols, using radioactively labelled bacteria [87].

Both homologous and heterologous antigen attracted equal numbers of neutrophils. Also, in the two different glands, the same proportions of neutrophils carried cytophilic antibodies. There was no suggestion that the neutrophils recruited by the homologous antigen had received a boost to their phagocytic activity as a result of any specific cytophilic antibody.

Smith et al. were reluctant to conclude that the cytophilic antibody made no difference at all in the attraction of neutrophils by the homologous antigen. In vitro studies had shown that neutrophils from immunized animals migrate along concentration gradients of antigen by virtue of interactions between cytophilic antibody and antigen [40]. Smith et al. hypothesized that neutrophil chemotaxis mediated through cytophilic antibody might stimulate short-range locomotion without being involved in recruitment of subpopulations of cells from blood.

Summary of exudate neutrophils

In conclusion, the behavior of neutrophils in an exudate is not easy to predict. The activity of exudate neutrophils seems to depend greatly on the character of that specific exudate fluid and on the chemotaxins to which the neutrophils have been exposed. Evidence certainly exists which

indicates that exudate neutrophils may respond differently to chemotaxins; they may be deactivated, adapted, or primed to respond to a specific chemotaxin. The activity of the superoxide-generating system in an exudate neutrophil may differ from that of a neutrophil in the peripheral circulation. A neutrophil may have experienced partial degranulation, especially of specific granules during its movement into the exudate. Further, although cytophilic antibody has been shown to affect neutrophil migration in vitro, it does not appear to be a major factor in movement of neutrophils within the body.

EFFECTS OF *ESCHERICHIA COLI* HEAT-STABLE ENTEROTOXIN B ON SMALL INTESTINAL VILLI IN PIGS, RABBITS, AND LAMBS

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Abstract

Culture supernates from two strains of *E. coli* were placed into different ligated intestinal sections (loops) of each animal. The two bacterial strains were identical except that one contained a plasmid carrying the heat-stable toxin b (STb) gene, while the other did not. Morphometric techniques were used to assess villous epithelial surface areas and mucosal volumes in both intestinal segments exposed to STb-positive (test) and to Stb-negative (control) supernates. In pigs whose intestines were exposed to STb-positive supernatants for 2 hours, both villous epithelial surface area and mucosal volume were significantly smaller in test loops than in control loops ($P<0.02$). In test loops of pigs incubated for 1 hour, and in test loops of lambs incubated for 2 hours, there was a decrease in villous epithelial surface area which approached the test for significance but did not meet it ($0.05<P<0.10$). Rabbit test loops did not differ from rabbit control loops in either villous epithelial surface area or mucosal volume. Histological examination of the tissues from all three species revealed epithelial changes in porcine and ovine tissues only. In porcine and ovine tissues, epithelium at villous tips was seen to be cuboidal or squamous, or even to be absent. Villi with similarly altered epithelium were seen in control loops, but were seen much more frequently in test loops. These epithelial changes were seen as early as 30 minutes of incubation in

pigs. Intestinal tissues from these pigs were examined by transmission electron microscopy, but no difference between test and control tissues was seen. We conclude that STb is capable of causing partial villous atrophy in young pigs after only 2 hours.

Introduction

Certain strains of Escherichia coli are a major cause of disease in domestic animals. In young animals, E. coli is associated with septicemia and, more often, with neonatal diarrhea. The diarrheal disease referred to as colibacillosis is debilitating and frequently fatal in calves, lambs, and pigs.

Mechanisms by which E. coli strains cause diarrhea have been investigated [6,14,15,17]. Several enterotoxins are elaborated by different strains of E. coli. One of these enterotoxins has been designated heat-stable toxin b (STb) [2]. Estimates of the field incidence of STb have been made by surveying collections of E. coli strains. A North American survey based on gene probe hybridization found that the STb gene was present in 75% of the enterotoxigenic E. coli (ETEC) strains [12]. A Brazilian survey using the pig loop assay found that 12.5% of the strains surveyed produced STb. [4]. This latter survey may underestimate the true incidence of STb, as E. coli strains which were positive for another enterotoxin were not tested for STb.

Escherichia coli enterotoxins including STb are thought to cause secretory diarrhea without causing discernible morphological damage to enterocytes [8]. However, the findings of Whipp and coworkers [22] suggested

that STb causes villous atrophy in ligated intestinal loops of 3-week-old pigs. In fact, more recent work by Whipp et al. confirms villous atrophy due to STb in 3-week-old pigs [23]. The work described here differs from this latter report in that a more objective measurement system is used to assess villous atrophy. Also, more species are exposed to STb, effects of STb are described at several incubation times, and tissues are examined with transmission electron microscopy.

The histological effects of STb on intestinal loops of three species were examined using two strains of E. coli (HB101 and HB101-pRAS-1), which are identical except that the latter strain carries a plasmid containing the gene encoding for STb [9]. It was anticipated that pigs would respond to STb with an inteestinal secretory response but that lambs and rabbits would not. (Although an early report in the literature stated that rabbits respond to STb with intestinal secretion [2], more recent work has indicated that they do not [8].) These three species were observed for morphological changes. Morphological effects of STb were evaluated using morphometric techniques [5], histopathology, and transmission electron microscopy.

Materials and Methods

Experimental animals were ten lambs (3 to 9 days old), 10 rabbits (60 to 74 days old), and 20 pigs (14 to 21 days old). Animals were held off feed for 24 hours. They were then anesthetized with Halothane and ligated intestinal sections (loops) were created by a method previously described [13]. Four loops were created in each animal; loops were 10 cm

long and were separated by interloops 2 to 4 cm long. In pigs, the first loop was created 100 cm distal to the ligament of Trietz, and subsequent loops were created distal to the first. In rabbits, the first loop was created just proximal to the ileocecal ligament, and subsequent loops were created proximally to the first. In lambs, the duodeno-jejunal flexure was identified, and the first loop was created 30 cm distal to that.

Test loops and control loops (two each) alternated with each other along the length of the intestine. Test loops were injected with a cell-free culture supernate from a heat-stable toxin b (STb)-positive bacterial culture; control loops were injected with a cell-free culture supernate from an STb-negative bacterial culture.

After surgery, animals were allowed to recover from the anesthesia. All lambs and rabbits were euthanized 2 hours after injection of their intestinal loops. Of the 20 pigs, five were euthanized at 15, 30, 60, and 120 minutes after creation of their loops.

Bacterial strains and culture conditions

Two strains of E. coli were used: HB101 (STb-negative) and HB101-pRAS-1 (STb-positive). The first strain was nonenterotoxigenic. The latter strain differed from the first only in that it contained a recombinant plasmid with the cloned gene for STb. The plasmid, pRAS-1, is derived from pBR322 and contains an insert which encodes for STb [9]. These two strains were inoculated separately in 10 ml of brain heart infusion (BHI) broth and incubated overnight at 37° C. Only 0.5 ml of

this material was used to inoculate 400 ml of BHI broth in a 2,000 ml Erlenmeyer flask. This flask was incubated fro 20 hours at 37° C while being agitated on a platform shaker at approximately 240 rpm. Bacterial cells were removed from cultures by centrifugation (15,300 x g, 25° C, 30 minutes). Culture supernates were filter sterilized (0.22 micrometers) and were stored at 4° C. These two preparations (one containing STb and one without) were used throughout the study.

A trace amount of the non-absorbable marker polyethylene-1,2-^{14}C-glycol (PEG-^{14}C)(mol. wt. approximately 4,000) was added to each of these two sterile filtrates (12 microcuries/liter) for measurement of fluid accumulation as described below.

Surgeries, necropsies, and samples

At the end of each animal's incubation period, it was anesthetized again with Halothane. The ligated loops were exteriorized; loop contents were aspirated. Volume of contents was recorded, and a small amount was reserved for PEG-^{14}C determination. The amount of PEG-^{14}C in the inoculum and the amount of loop fluid at the end of the incubation time were used to calculate fluid movement into or out of each loop [21]. For each animal, control loop volumes were subtracted from test loop volumes. The resulting value was a measure of loop response to STb. A zero-time volume of fluid in each loop was determined at the time of injection of supernate; a small amount of fluid was withdrawn after injection, and the concentration of PEG-^{14}C was determined.

In each animal, one control loop and one test loop were injected with

2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4). Just enough fixative was injected into the lumen of the loop so that fixative was in contact with all sides of the loop. The other control and test loops were similarly injected with 10% buffered neutral formalin. Loops were excised and placed in their respective fixatives. The animal was euthanized by an overdose of succinylcholine.

Formalin-fixed loop tissues were first samples so that three transverse slices of intestine were available for processing. One slice came from the center of the loop, and the other two were near the ends of the loop. Transverse slices were dehydrated, embedded in paraffin, cut into 3-micrometer sections, and stained with hematoxylin and eosin (H&E). All three sections from a loop were placed on the same slide. Resulting histological sections were encoded and evaluated by morphometric techniques (see below) and for histologically detectable lesions. All the villi in a transverse section were examined, and the percent of villi showing abnormalities was recorded. An abnormality was defined as cuboidal epithelium or a gap in the epithelium. Villi with vacuolated epithelial cells were also noted although these vacuolated cells were considered normal.

A central portion of each glutaraldehyde-fixed loop was cut into 1 mm cubes and fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 1 hour. Tissues were then transferred to 0.05 M sodium cacodylate buffer (pH 7.4) overnight. These pieces of tissue were post-fixed with 1% osmium tetroxide for 2 hours after which they were rinsed

again in the cacodylate buffer for 1 hour. They were dehydrated in a series of graded ethanols and cleared in propylene oxide. The tissues were embedded in Medcast resin (Pelco, Tustin, CA). Ultrathin sections (70 to 90 nm) were stained with uranyl acetate and lead citrate. They were examined with a Philips 410 transmission electron microscope operated at 60 kilovolts.

Morphometry

Morphometric techniques described by Chalkley, Cornfield, and Park [3] and by Weibel [19] were used. The same type of grid used by Dunnill and Whitehead [5] in their examination of intestinal tissue was used (Fig. 1). The ends of the lines represent points. When points fell within the intestinal mucosa, they were referred to as "hits." When the lines themselves intersected intestinal epithelium, these intersections were referred to as "cuts." Briefly, points are proportional to volume, and cuts are proportional to surface area. When the grid was placed over each new field, one line was positioned so that it crossed the muscularis mucosa. Dunnill and Whitehead's method of sampling was followed; each loop was examined until 200 hits had been recorded (approximately 70-3-micrometer section). The number of fields examined before counting 200 hits was recorded. Villi over Peyer's patches were not examined because the mucosal volume was altered there due to lymphoid tissue. Once the slides had been analyzed and decoded, the data for each loop were compiled and were expressed as cuts/field and hits/field.

Fig. 1. Grid of 15 lines of equal length superimposed on transverse section of intestine. h = hit; c = cut

Statistical analysis utilized the Student's t-test and a determination of correlation coefficient (r) [18]. To minimize the effect of animal-to-animal variation, differences between test and control loops were analyzed, rather than test and control values themselves.

Results

All groups of pigs whose loops were allowed to incubate for 30 minutes or longer showed a significantly ($P<0.05$) greater amount of fluid accumulation in heat-stable toxin b (STb)-treated loops, as did the lambs (Fig. 2). Rabbits had approximately 0.5 ml less in STb loops than they did in control loops ($P<0.05$). We concluded that the test inoculum contained STb. The pigs were the last group of experimental animals; the one preparation of STb-positive culture supernate had maintained STb activity throughout storage.

Three out of 20 pigs did not respond to STb with secretion in their test loops. Loops of one such pig were exposed to the culture supernates for 2 hours; the loops of the other two pigs were exposed for 1 hour. Five out of ten lambs also did not respond with secretion in test loops. Experiments in our lab have occurred before in which 50% of pigs tested did not respond to STb; hence these non-responding animals were not considered to be unusual [20].

Morphometric analysis revealed that villous epithelial area (cuts/field) was reduced in porcine loops exposed to culture supernates of STb+ bacteria for 2 hours ($P<0.02$) (Fig. 3). Epithelial surface area in test loops of these animals was approximately 75% of the area in

Fig. 2. Loop response to heat-stable toxin b (STb). Value for each category represents the difference in ml between test loops and control loops (mean + standard error). A positive value means that test loops contained more fluid than did control loops at the end of the incubation time

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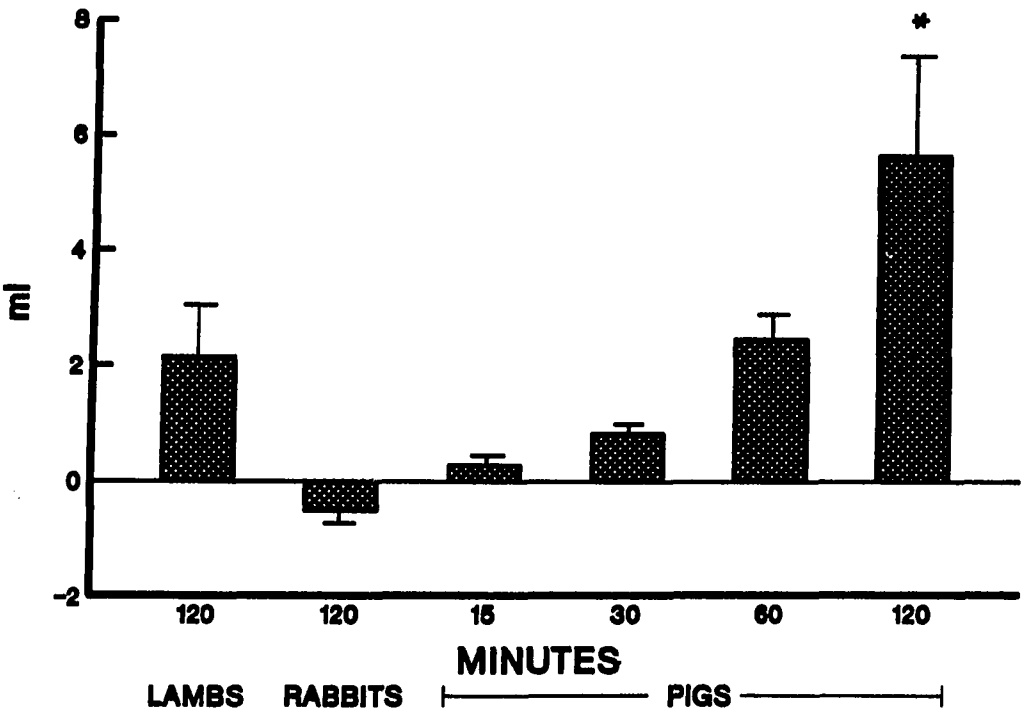
*Treatment differs from control, $P < 0.05$


8
6
4
2
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-2
ml
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120
120
15
30
60
120
MINUTES
LAMBS
RABBITS
PIGS

Fig. 3. Cuts/field. Treatment and control values. Cuts/field is a measurement of villous epithelial area

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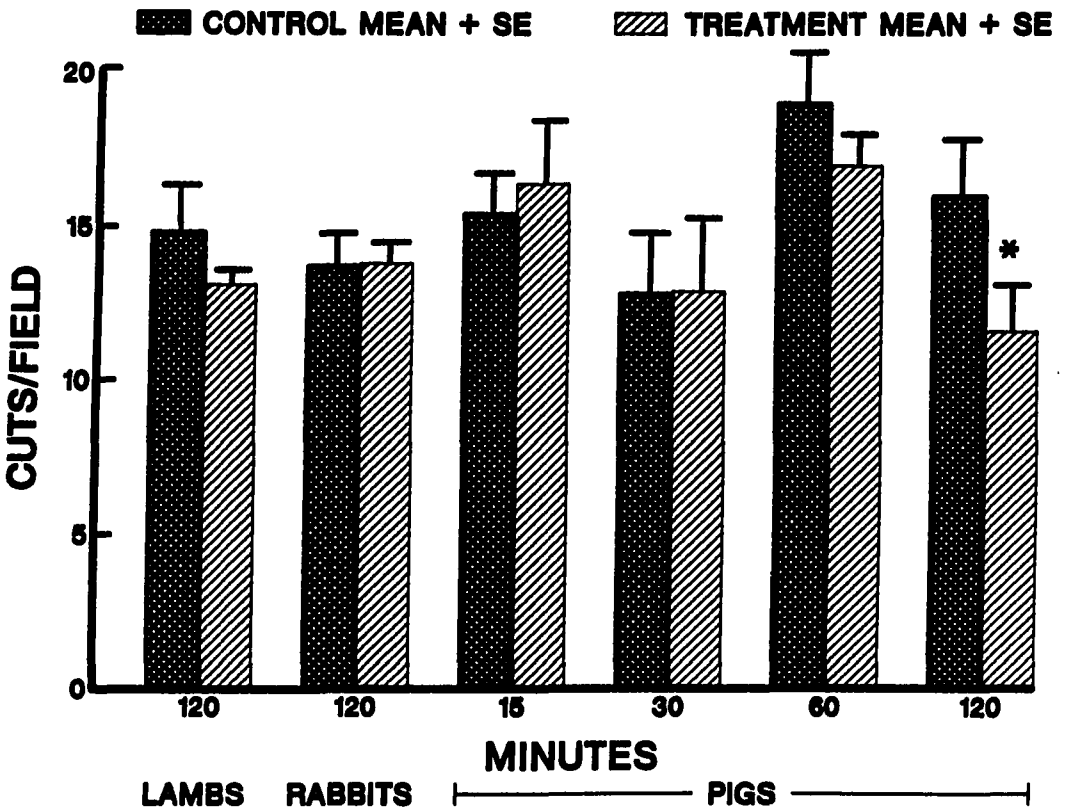
*Treatment differs from control, $P < 0.02$


CONTROL MEAN + SE
TREATMENT MEAN + SE
CUTS/FIELD
20
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5
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120
120
15
30
60
120
MINUTES
LAMBS
RABBITS
PIGS

control loops. The decrease in epithelial surface area of both ovine loops exposed to STb for 2 hours and of porcine loops exposed to STb for 60 minutes just failed the test for significance ($0.05<P0.10$).

Mucosal volume was also reduced in porcine loops exposed to STb for 2 hours ($P<0.02$)(Fig. 4). The mucosal volume in test loops was approximately 80% of that in control loops.

Data from pigs whose loops were exposed for 1 or 2 hours and from lambs whose loops were exposed for 2 hours were examined. These were the groups of animals in which villous changes (decrease in epithelial surface area or mucosal volume) in STb+ loops either were significant or approached significance. Although these groups of animals as a whole responded to STb with secretion in test loops, not every animal responded with secretion. Among the responders, some animals secreted more fluid into test loops than did others. There was also variation between animals in the degree of decrease in villous epithelial area or mucosal volume. For each animal, the difference in fluid between test and control loops was compared with two other values; the difference in cuts/field between test and control loops, and the difference in hits/field between test and control loops. There was no correlation between fluid distention and a decrease in either villous epithelial area or mucosal volume. The r values ranged from -0.03 to +0.44 for the different groups.

Fig. 4. Hits/field. Treatment and control values.

Hits/field is a measurement of mucosal volume

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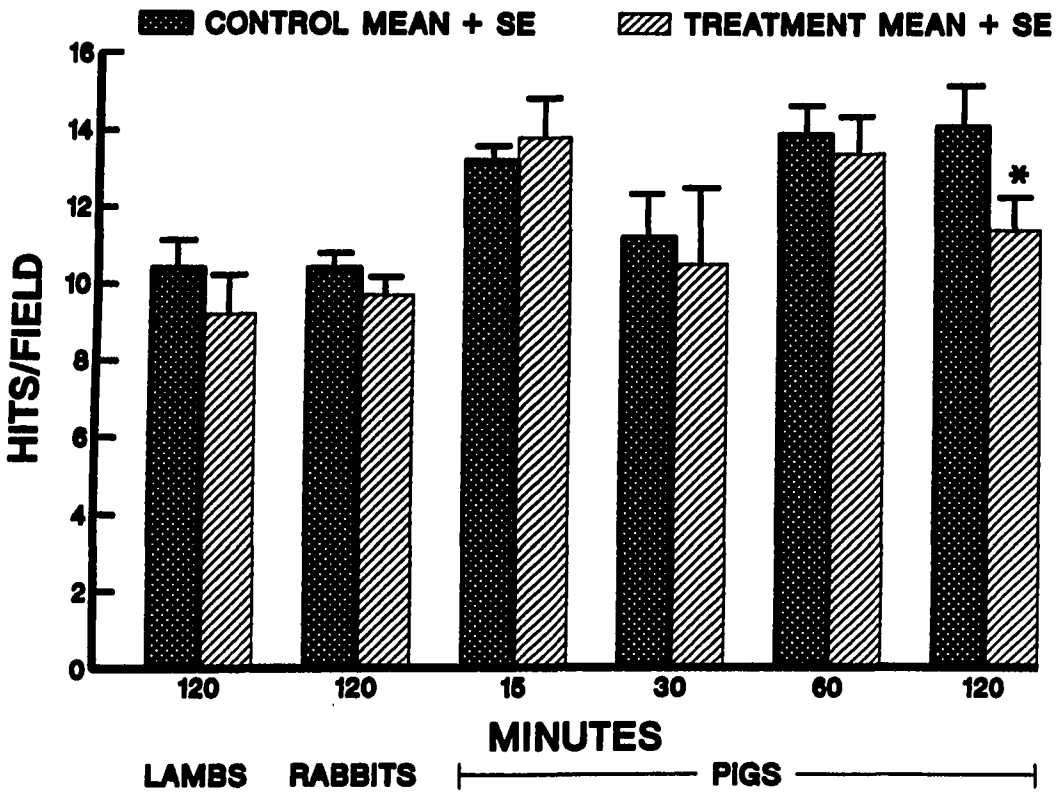
*Treatment differs from control, P< 0.02


CONTROL MEAN + SE
TREATMENT MEAN + SE
HITS/FIELD
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14
12
10
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0
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120
120
15
30
60
120
MINUTES
LAMBS
RABBITS
PIGS

Data from animals which were "strong secretors" (six pigs and four lambs) were examined. Secretion was accompanied by a decrease in epithelial surface area, or mucosal volume, or both.

Tissues of all three species were examined histologically. Two types of abnormalities in villous epithelium were detected (Table 1): cuboidal to squamous epithelium, and gaps in the epithelium. These abnormalities were not present in rabbit tissues. Most of the abnormal villi were found in the lamb tissues and in the porcine tissues which had been exposed to the culture supernates for 30 minutes or longer. Although some villi with abnormal epithelium were found in control tissues, most were found in test tissues. On some villi, the epithelium appeared completely normal except for a focus of four to five cells in which the cytoplasm was highly vacuolated (these foci are possibly remnants of vacuolated epithelial cells seen in neonatal pigs) [11]. On other villi, epithelium was cuboidal or even squamous, rather than columnar. Some villi were partially denuded. That is, there was an appreciable gap of three to five cells' width in the villous epithelium. The remaining epithelium on such villi was cuboidal (Fig. 5).

Tissues from pigs whose intestinal loops were exposed to bacterial supernatants for 30 minutes or for 2 hours were examined by transmissionelectron microscopy. Tissues from the pigs allowed to incubate for 2 hours were chosen because the morphometric analysis revealed villous atrophy in test loops. Tissues from the pigs allowed to incubate for 30 minutes were chosen to find early cellular changes. In

Table 1. Histologic analysis of villi. [a]

Experimental Animals (n = loops)	Normal Villus (non-vacuolated)	Normal Villus (vacuolated)	Villus with Cuboidal Epithelium	Villus with Gap in Epithelium
Pigs 15 min				
Control (5)	81	16	2	1
Test (5)	83	15	1	1
Pigs 30 min				
Control (5)	86	4	10	1
Test (5)	58	1	33	8
Pigs 60 min				
Control (5)	94	0	6	1
Test (5)	86	1	13	1
Pigs 120 min				
Control (5)	84	6	9	1
Test (5)	64	1	31	5
Rabbits 120 min				
Control (10)	100	0	0	0
Test (10)	100	0	0	0
Lambs 120 min				
Control (10)	85	1	13	1
Test (10)	69	1	28	2

[a]Numbers in table refer to percentage of villi examined. Three intestinal sections per loop were analyzed. All villi were examined and each villus was put into one of four categories.

Fig. 5. Histological section of porcine intestinal loop exposed to E. coli STb+ supernate for 2 hours

both sets of tissues, degenerative changes were seen in tissues exposed to STb-positive supernatants; the same changes could be found in tissues exposed to STb-negative supernatants (Figs. 6-8).

In porcine tissues from the 2 hour incubation group, within the epithelium, intercellular spaces were expanded with fluid and usually contained cellular debris. A majority of mitochondria contained intramitochondrial dense bodies. In addition, mitochondrial matrix was flocculent in about 20% of the mitochondria. Areas of rarefaction were noted in the matrix of about 50% of the mitochondria. Rough endoplasmic reticulum was distended in about one-half of the epithelial cells. Nearly every epithelial cell contained at least one autophagic vacuole. At the luminal surface, blebbing of cytoplasm or shortened and irregular microvilli were seen in a very few epithelial cells. Even fewer cells had dark, condensed cytoplasm.

In these same tissues, the lamina propria in both STb-positive and STb-negative tissues was edematous and contained cellular debris. About one-fourth of the cells in the lamina propria contained autophagocytic vacuoles.

In the porcine tissues from the 30 minute incubation group, similar changes were again seen in both test and control tissues. Epithelial changes were mild: expanded intercellular spaces and occasional dilatation of rough endoplasmic reticulum. Although some cellular debris was noted in the lamina propria immediately subjacent to the epithelium,

Fig. 6. Epithelium from porcine intestinal loop exposed to STb-negative bacterial supernatant for 2 hours. Exaggerated intercellular space containing lymphocytes. Similar profiles seen in test loops

Fig. 7. Epithelium from a porcine intestinal loop exposed to STb+ bacterial supernate for 2 hours. Numerous autophagocytic vacuoles. Similar profiles seen in control loops.

Fig. 8. Epithelium and lamina propria from a porcine loop exposed to STb+ bacterial supernate for 2 hours. Wide intercellular spaces in epithelium. Similar profiles seen in control loops

there was less debris than was noted in the 2 hour tissues. Vessels and crypts were normal in both sets of tissues.

Discussion

This study demonstrates that villous atrophy in pigs can be due to the enterotoxin STb. Although villous atrophy in both pigs and calves has been noted in field and experimental cases of enterotoxigenic E. coli infection [1,7,16] it was not certain what enterotoxin or other toxin was causal.

Our results support the idea that a species' ability to secrete in response to STb is a prerequisite for induction of damage by STb. No secretory response occurred in rabbits, and no histologic damage was present. Secretion and histologic damage were both seen in pigs. Although the situation is less clear in lambs, the trends observed indicate that STb can induce secretion and histologic alterations in sheep. That secretion and histologic damage are not inextricably linked in an individual animal is evident in that STb-induced morphological changes have been observed in pig loops in the absence of secretion [20].

The histological lesions in lamb intestines exposed to STb for 2 hours and in pig intestines exposed to STb for 30 minutes or more are those one would expect to see with villous atrophy due to enterocyte damage, e.g., cuboidal epithelium and partially denuded villi.

Transmission electron microscopy did not reveal a mechanism by which STb caused villous atrophy. No detectable differences between the STb-exposed tissues and the control tissues were seen. Epithelial cells

damaged by STb may have already sloughed from the villous surface. Those cells remaining on the villous surface were no more or less degenerate than those cells in the STb-negative tissues.

The villous atrophy in test loops of pigs with 2 hour incubation periods was not an artifact caused by loop distention, as there was no correlation between degree of distention and degree of villous atrophy. However, we did not see secretion which was not accompanied by some level of villous atrophy. Secretion did not occur in the absence of lesions.

Our use of morphometry was not to measure absolute villous epithelial surface area or mucosal volume. Rather, we wanted to follow changes in these parameters and to see if they changed in concert with one another. In our grid, in which lines are oriented at 0^{o}m 120^{o}, and 240^{o}, rotation of line compensates for isotropy in a tissue. In a transverse section of gut, villi have no preferential alignment, and can thus be considered anisotropic [10]. Cross-sections of intestine were not always circular (some were elliptical), but the variance of the line angle compensated for that.

We have demonstrated that the E. coli enterotoxin STb will cause villous atrophy in the small intestine of pigs after 2 hours of incubation. This villous atrophy may be occurring in lambs exposed to STb for 2 hours and in pigs exposed for 1 hour. Also, 1-week-old lambs can respond to STb with intestinal secretion.

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ELICITATION OF ENTEROLUMINAL NEUTROPHILS BY ENTEROTOXIGENIC AND NONENTEROTOXIGENIC STRAINS OF *ESCHERICHIA COLI* IN SWINE

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ELICITATION OF ENTEROLUMINAL NEUTROPHILS BY ENTEROTOXIGENIC AND NONENTEROTOXIGENIC STRAINS OF *ESCHERICHIA COLI* IN SWINE

Abstract

In intact neonatal piglets, two strains of enterotoxigenic *Escherichia coli* (ETEC), which could adhere to and colonize the small intestine, attracted greater numbers of neutrophils into the lumen and wall of the intestine than did a nonenteropathogenic strain of *E. coli*. Ligated loops of small intestine in 8-week-old pigs were used in attempts to identify the attributes of ETEC involved in stimulating an increased enteroluminal migration of neutrophils. A nonenteropathogenic strain of *E. coli* did not attract neutrophils into the intestinal lumen in this model. However, three of the five ETEC strains tested did so. The three positive strains all produced heat-stable enterotoxin type b (STb). Neither of the negative ETEC strains produced STb. An STb-containing culture supernatant prepared from a strain of *E. coli* which contained an STb plasmid did not attract significantly more neutrophils than did a control supernatant prepared from the same strain of *E. coli* without the plasmid. The ETEC strains which attracted neutrophils in loops did not associate intimately with loop villi more consistently, nor did they grow to higher numbers in loops than did strains which did not. It was concluded that there are increased numbers of neutrophils in the intestinal lumen during ETEC infection of newborn pigs. However, attempts to identify the attribute(s) of ETEC responsible for eliciting enteroluminal neutrophils were not successful.

Introduction

Neutrophils are associated with many types of intestinal infections. Tissue damage generated by invasive bacteria, viruses, or parasites attracts neutrophils [12]. Increased numbers of neutrophils in the lumen of the gut in calves suffering from enterotoxigenic Escherichia coli (ETEC) infection have also been described [2]. The histopathological lesions in these calves were relatively mild (villous blunting, focal degeneration of enterocytes, and exfoliation of epithelial cells), yet emigration of neutrophils into the lumen was observed. A noninvasive intestinal disease such as that caused by ETEC would presumably attract neutrophils into the lumen of the gut by methods other than gross tissue injury. The chemotactic agents which are effective in such a noninvasive disease are unknown. Beneficial effects of the enteroluminal neutrophils to the host have been proposed [1-3,11,20], although others feel that neutrophils in the intestine are deleterious [9].

Bellamy and co-workers [1-3] and Sellwood [20] suggested that neutrophils play a protective role in the intestinal lumen. Bellamy and Nielsen developed the concept of immune-mediated emigration of neutrophils into the lumen of the gut [3]. According to Bellamy, the presence of an antigen in the lumen of the gut would attract neutrophils, provided that the host possessed circulating specific antibody. He also showed that the presence of enteroluminal neutrophils was associated with a luminal bacterial population which did not proliferate; he believed that phagocytosis on the mucosal surface was an important protective mechanism

in the small intestine [1]. We tried to duplicate the immune-mediated chemotaxis experiments of Bellamy but were unable to do so for reasons that are as yet unclear. The concepts and results presented by Bellamy and co-workers merit additional study.

Sellwood collected immune and nonimmune colostrum, monitored the *in vivo* protection such colostrum gave to piglets [20], and concluded that immune colostrum could function *in vitro* by preventing adhesion of the bacteria to gut epithelial cells and by promoting phagocytosis. He also proposed that these mechanisms were active *in vivo*, provided that emigration of neutrophils across intestinal epithelium occurs in newborn piglets.

The objectives of the work reported here were (i) to determine whether the presence of *E. coli* causes neutrophils to emigrate into the intestinal lumen of intact newborn piglets; (ii) to determine whether *E. coli* strains vary in their ability to attract neutrophils into the lumen of ligated intestinal loops in swine; and (iii) to define some of the attributes of *E. coli* that are involved in attracting neutrophils into the intestinal lumen.

We report here difference between ETEC and non-ETEC strains in the attraction of enteroluminal neutrophils in neonatal piglets. In ligated loops, these strain differences are not attributed to any chemoattractant activity of enterotoxin.

Materials and Methods

Four different experiments were performed (Table 1). Reference is made to these in the text below when appropriate.

E. coli strains and preparations

Pertinent characteristics of the strains of E. coli used are listed (Table 2). Each strain of E. coli was grown by inoculating 10 ml of Trypticase soy broth (TSB;BBL Microbiology Systems, Cockeysville, Md.) or brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) and shaking for 18 h aerobically at 37° C. The only strains inoculated into BHI were those cultured specifically for heat-stable enterotoxin b (STb) production (see below). Supernatants required were prepared by centrifuging the bacterial growths at 15,300 x g for 30 minutes at room temperature, followed by filtering them through a membrane filter (pore size, 0.22 microns; Millipore Corp.).

Five separate cultures of E. coli 263 were killed by five different methods: application of moist heat, Beta-propiolactone, mercurochrome, phenol, or Formalin. The culture killed by moist heat was exposed to steam for 65 min. Another culture of strain 263 was made 0.2% Beta-propiolactone and allowed to set for 10 min before being washed with TSB. Killing with mercurochrome was done by first centrifuging a culture of strain 263 as described above. The supernatant was decanted, and 6 ml of mercurochrome was added to the pellet and allowed to react for 1 h. The solution was then centrifuged again, and the pellet was washed three

Table 1. Different experiments performed

Expt. no.	Experimental animals (status of intestine)	n	Question
1	Neonatal piglets (intact intestine)	15	Do ETEC and non-ETEC strains attract neutrophils into the intestinal lumina of neonatal piglets?
2	8-week-old pigs (ligated loops)	6	Will all strains attract neutrophils in a system in which they all attain high numbers? Will killed E. coli cells attract neutrophils?
3	8-week-old pigs (ligated loops)	8	Do positive and negative strains attain similar numbers in loops?
4	8-week-old pigs (ligated loops)	6	Is STb an attractant?

Table 2. Characteristics of *E. coli* strains used

Strain	Serotype	Enterotoxin
123	O43:K-:H28	None
263	O8:K87,K88:H19	LT, STb
431	O101:K30(A),K99:NM	STa
987	O9:K103,987P:NM	STa, STb
A-1 (1288)	O149:K91,K88:H10	LT, STa, STb
Troyer (1459)	O9:K35,K99:NM	STa
HB101	K12 (rough)	None
HB101 (pRAS-1)	K12 (rough)	STb

times in sterile TSB. The final pellet was resuspended in 6 ml of TSB. Killing with 3% phenol or 10% Formalin was done by the mercurochrome procedure. The effectiveness of these treatments in killing the bacteria was verified by plating.

Intact neonatal piglets (Experiment 1)

Fifteen neonatal piglets delivered by Caesarean section and deprived of colostrum were used. Each piglet was given 20 ml of pooled normal pig serum intraperitoneally [24]. Nine of the piglets each received an oral inoculum of 8×10^9 cells of E. coli 123, 431, or 987 at 6 to 8 h of age. The remaining six control piglets received only 5 ml of TSB. One pig per E. coli strain and two control piglets were necropsied at 2, 8, and 16 h postinoculation.

At necropsy, two locations were chosen for fluid sampling. One location was in the ileum, 5 cm proximal to the ileocecal ligament. The other location was at the tip of the spiral colon. (This fluid was sampled to find neutrophils washed down from the small intestine.) A piece of intestine approximately 5 cm long was ligated off at each location. Saline (2 ml) was injected into each of these loops and then withdrawn. Smears of this saline flush were fixed and stained for peroxidase-positive cells as described below. The smears were then examined microscopically to determine the percentage of neutrophils present in the cells of this flush.

Tissue samples were taken at the upper jejunum (5 cm distal to the ligament of Trietz), the ileum, and the mid-small intestine (lower

jejunum). These were processed and examined microscopically for neutrophils and layers of adherent bacteria.

Intestinal loop procedures (experiments 2 to 4)

Eight-week-old pigs were fasted overnight. General anesthesia was induced and maintained with halothane. A total of 6 to 14 ligated intestinal loops were created in each pig by a method previously described [15]. Each loop was 10 cm long; interloops were 2 to 4 cm long. The first loop was created 100 cm distal to the ligament of Treitz, and subsequent loops were created distally from the first. The location of a particular inoculum was varied from pig to pig, so that a particular inoculum did not always occur at one location within the intestine.

Inocula were injected with a 26-gauge needle. Each inoculum contained 6 ml of test material plus 3 ml of 320 mOsM mannitol. The test material was either an overnight TSB culture of E. coli (first six strains in Table 2) or a supernatant from a BHI overnight culture of the E. coli (last two strains in Table 2; see STb preparation below). The mannitol ensured that fluid would remain in the intestinal segment.

After the last ligated loop had been inoculated, the small intestine was returned to the abdominal cavity. The abdominal wall was closed, and the animal was allowed to recover from surgery. The pigs were killed at either 2, 4, or 6 h after surgery by injection of sodium pentobarbital IV. Sample collection is described below. After the samples were collected, the rest of the pig carcass was inspected for the presence

of any complicating factors, such as purulent pneumonia, which might competitively attract neutrophils. No such factors were found.

Fixing, staining, and identifying neutrophils in smears of intestinal fluids (experiments 1, 2, and 4).

The smears were allowed to air-dry and were then fixed for 1 min in a solution of 1 part 10% buffered neutral formalin-3 parts 96% ethanol. The fixed smears of intestinal fluid were stained for peroxidase-positive cells [25]. Fixed smears were rinsed in distilled water and counterstained with 1% toluidine blue.

Microscopically, neutrophils appeared as lobulated blue nuclei associated with fine brown granules. Eosinophils were distinguished by their very large brown peroxidase-positive granules. Many of the cells observed were larger than neutrophils, had round nuclei, lacked granules, and were assumed to be mostly epithelial cells. Two hundred cells were examined from each smear, and the percentage of cells that were neutrophils was recorded.

Total neutrophils per loop (experiment 2)

Mucolexx (Lerner Laboratories, New Haven, Conn.) (1 ml) was added to each 4.5-ml sample of intestinal fluid to facilitate the use of a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). The volume of fluid present in each ligated loop was recorded. The number of cells per milliliter was converted to cells per loop, taking into account the addition of Mucolexx. Multiplication of the number of cells per loop by

the percentage of neutrophils in that loop yielded a value for the total number of neutrophils per loop.

Processing of solid tissues (experiments 1 to 4)

Tissues were placed in Bouin-Hollande fixative immediately after necropsy. This fixative is similar to Bouin fixative [21], except that it is also made 2.5% cupric acetate. The tissues remained in this fixative for 18 h. They were washed three times in 70% ethanol and then three times in 50% ethanol. These tissues were dehydrated, embedded in paraffin, sectioned at 3 micrometers, and stained with hematoxylin and eosin. All histological sections (two per loop) were encoded, evaluated microscopically by the following criteria, and decoded, and the results were summarized. The criteria for grading the sections are: 0, neutrophils not present in section; +, neutrophils are occasionally present in villi; ++, neutrophils are seen consistently in subepithelial capillaries, within the epithelial cell layer, and within the lamina propria of the villi; +++, neutrophils are plentiful in the above areas, and the epithelium at villous tips is occasionally absent; ++++, criteria for (+++) are present, the epithelium at villous tips is consistently absent, and a crust of neutrophils on the villi is at least 15 neutrophils thick.

Duplicate sections of ligated loops from experiment 2 were stained with a Brown-Hopps modified Gram stain [21] to facilitate microscopic evaluation of bacteria adherent to intestinal epithelium. Adherence was determined as the presence or absence of bacterial layers on the villi.

Evaluation of numbers of bacteria (experiment 3)

To evaluate the effect of bacterial proliferation on the ability of E. coli to elicit neutrophils into the lumina, seven ligated loops were created in each of eight 8-week-old pigs. Each loop was inoculated with 2.5×10^7 bacteria (one loop for each of the first E. coli strains shown in Table 2) or with TSB. The different strains were rotated in the different pigs in terms of location within the intestine. Four pigs were allowed to survive for 4 h after intestinal loop surgery; four were allowed to survive for 6 h.

At the time of necropsy, each loop was collected aseptically. The tissue and fluid from each loop were homogenized with a Virtis 45 homogenizer (The VirTis Co., Inc., Gardinar, N.Y.), and the volume of the homogenate was recorded. The concentration of viable bacteria in each homogenate was determined by using a spiral plater (Spiral Systems, Inc., Cincinnati, Ohio).

The different loops were compared in terms of the number of bacteria per milliliter and the total number of bacteria per loop.

STb production (experiment 4)

Two strains of E. coli were obtained from S.L. Moseley. One strain, E. coli HB101, was nonenterotoxigenic. The other strain, E. coli HB101(pRAS-1), differed from the first strain only in that it contained a recombinant plasmid with the cloned gene for STb [13]. These two strains were inoculated separately in 10 ml of BHI and incubated overnight at 37° C. Only 0.5 ml of this material was used to inoculate 400 ml of BHI in a

2,000-ml Erlenmeyer flask. This flask was incubated for 20 h at 37° C while being agitated on a platform shaker at approximately 240 movements per min. Supernatant was prepared as described above.

STb loops and samples (experiment 4)

In each of six 8-week-old pigs, six ligated intestinal loops were created. Two loops were exposed to BHI, two others were exposed to supernatant from strain HB101, and the last two were exposed to supernatant from strain HB101(pRAS-1) (STb). The pigs were killed and necropsied 4 h after surgery.

The volume of fluid in each loop was recorded. Smears of this fluid were fixed and stained, and the percentage of cells that were neutrophils was determined. Tissues from three loops from each pig (one loop per treatment) were fixed as described above and were then graded for the present of neutrophils as described above.

Statistical methods

Levels of significance were determined by the Student's t-test [23, pp. 54-56].

Results

Intact neonatal piglets (experiment 1)

Strain 123 did not colonize the small intestine (form layers of bacteria adherent to villi), and the number of neutrophils in the mucosae and the percentage of neutrophils in the lumina of the intestines of pigs inoculated with strain 123 did not differ from those of the controls.

The other two strains (431 and 987) began to colonize the small intestine at 2 and 8 h postinoculation, respectively. Colonization was correlated with increased numbers of neutrophils in the intestinal mucosa and an increased percentage of neutrophils among cells in the lumen (Table 3).

In pigs which had visible adherent bacterial layers, neutrophils were clearly present in the mucosa of the small intestine (especially in the lower small intestine). They were consistently seen in the subepithelial capillaries, between epithelial cells, and in the intestinal lumen. The lumina contained knots of neutrophils and bacteria. Occasional small gaps in the epithelium were noted on the distal one-third of villi, and sometimes neutrophils appeared to be entering the lumen through such gaps.

In the intact neonatal piglets, we did not have a method for measuring the total neutrophils in the lumen of the gut. We tried to evaluate the total number of cells elaborated into the different guts on a relative basis by examining the total number of cells in the luminal smears. The smears which had a higher percentage of neutrophils also had markedly more total cells. Therefore, we concluded that an increase in the percentage of neutrophils in smears of luminal fluid reflected an absolute increase in the total number of neutrophils in luminal fluid.

Neutrophils attracted by different strains of E. coli in ligated intestinal loops (experiment 2)

Strains such as 123, which do not colonize intact pigs, can attain high numbers in ligated intestinal loops even though they do not adhere

Table 3. Relationship between colonization of intestine and presence of neutrophils in wall and lumen of intestine of neonatal piglets [a]

E. coli strain	No. colonized/ total no. [b]	Histological grading of neutrophils in intestinal wall [c]	Mean % of neutrophils in smears of luminal fluid [d]
None	0/12	0/12	2.0 (8)
123	0/6	1/6	0.3 (4)
431	4/6	6/6	25.8 (4)
987	5/6	6/6	36.5 (4)

[a]Data represent piglets necropsied at 8 and 16 h postinoculation.

[b]Number of samples with layers of bacteria adhered to villi. There were three samples per pig: upper jejunum, lower jejunum, and ileum. There were four control pigs and two pigs each for other treatments. Negative colonization values for strains 431 and 987 reflect noncolonization of the upper jejunum.

[c]Histological sections graded + or greater/number of sections examined. The one negative value for strain 987 was an upper jejunal sample.

[d]Number in parenthesis is the number of smears examined.

to villi. Intestinal loops also permit comparisons among several strains in the same animal. Each of the first six strains (Table 2) was tested at least once in ligated intestinal loops in each of six different pigs. Data for 4- and 6-h incubations were pooled to measure neutrophil response to a strain, because these data were of a similar magnitude. Data from the 2-h incubation were not included in this pool because responses at 2 h were uniformly minimal.

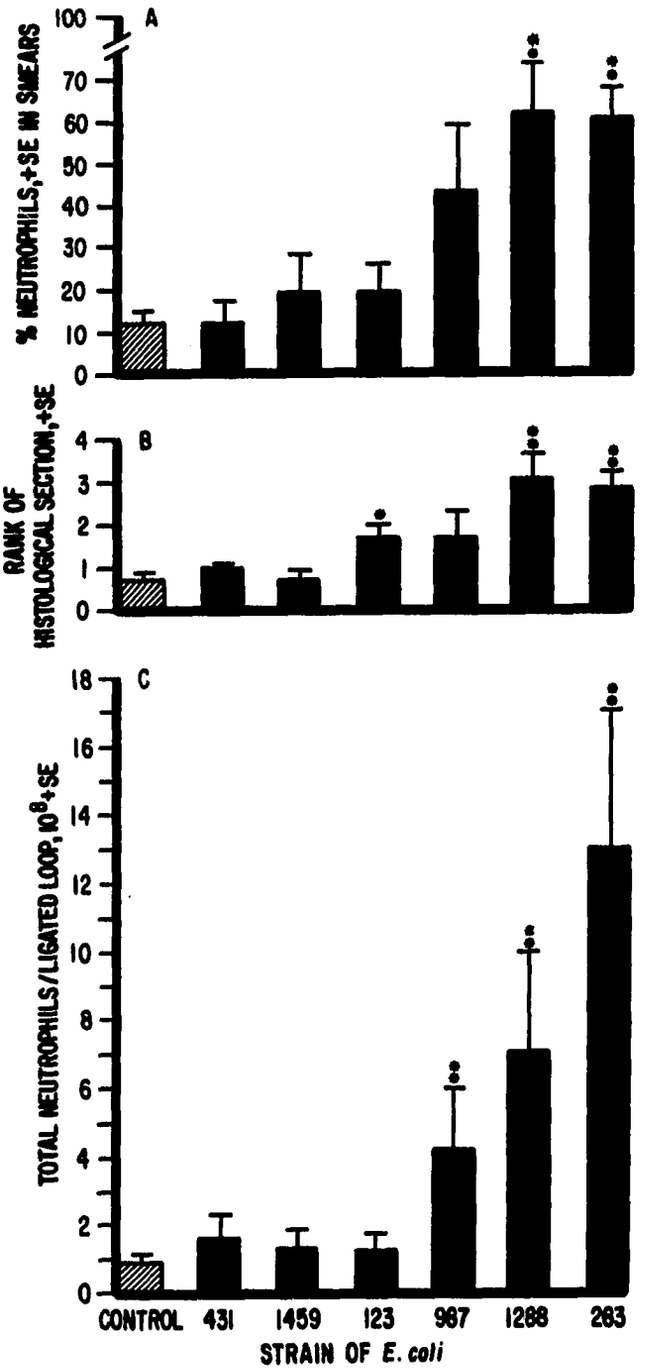
Different strains of E. coli were inoculated in a total of two to four replicate loops for 6 h of incubation and three to five replicate loops for 4 h of incubation. Loops containing only TSB and mannitol were replicated four times for 6 h of incubation and eight times for 4 h of incubation.

The first data to be compared were those of the percentage of neutrophils among smears of loop fluid. Figure 1 shows these means and their standard errors. When these means were compared, strains 263 and 1288 clearly attracted more neutrophils than did the control ($P < 0.01$). Values for the other strains did not differ from those for the controls.

The histological rankings for each strain were averaged and are also shown in Fig. 1. Strains 263 and 1288 again differed from the controls ($P < 0.01$), as did strain 123 ($P < 0.05$) (Fig. 1). The remaining strains did not differ from the controls.

When averages of total neutrophils per loop were compared by strain, strains 263, 987, and 1288 were found to be significantly different

Fig. 1. Neutrophils in ligated intestinal loops compared by *E. coli* strain in inoculum. Values are averages from 4- and 6-hour incubations combined. Each data point represents five to nine loops in a total of six pigs. (A) Percentage of neutrophils in smears of intestinal fluid; (B) grading of intensity of neutrophil infiltrate in histological sections; (C) total enteroluminal neutrophils per ligated loop. Statistically significant differences from the control value are as indicated: *, $P < 0.05$; **, $P < 0.01$


A
% NEUTROPHILS, +SE IN SMEARS
100
70
60
50
40
30
20
10
0
B
RANK OF HISTOLOGICAL SECTION, +SE
4
3
2
1
0
C
TOTAL NEUTROPHILS/LIGATED LOOP, 10^8+SE
18
16
14
12
10
8
6
4
2
0
CONTROL
431
1459
123
967
1288
283
STRAIN OF E. coli

from the controls ($P<0.01$) (Fig. 1). The remaining strains did not differ from the controls.

We also summarized data to examine changes over time for each strain. Only strains 263, 987, and 1288 yielded a significantly greater percentage of neutrophils after 6 h of incubation than they did after 4 h of incubation ($P < 0.05$).

Adherence of bacteria in loops (experiment 2)

We considered the possibility that bacterial adhesion was required for a strain to elicit neutrophils. For example, strain 431 caused a positive response in intact neonates where it adheres readily. It does not adhere well *in vitro* to cells from pigs of the age used for the ligated intestinal loop studies [18] and also did not attract neutrophils in these older pigs. For this reason, we reviewed Gram-stained histological sections from all the intestinal loops and scored them by using layers of bacteria associated with the villous epithelium as an index of adhesion [5]. There was no correlation between the occurrence of epithelial-associated bacteria and neutrophil response.

Comparison of live and killed *E. coli* cells (experiment 2).

Five different methods of killing *E. coli* 263 were used. All methods successfully killed the bacteria and all elicited similar numbers of neutrophils into ligated loops. Therefore, these data were pooled and referred to by "killed 263." The number of neutrophils elicited into a loop by killed 263 did not differ significantly from control values

(Table 4). However, the number of neutrophils elicited into a loop by live E. coli did differ from control values ($P < 0.01$).

Growth of bacteria in loops (experiment 3)

The mean number of E. coli cells per loop after 4 and 6 h of incubation for all six strains ranged from 2×10^8 to 8×10^8. The three strains of E. coli which attracted the most neutrophils into loops (263, 1288, and 987) in experiment 2 were not present in higher numbers than were the other three strains.

Chemotaxis and STb (experiment 4)

The three strains of E. coli which attracted the largest numbers of neutrophils into the lumen in ligated intestinal loops shared one factor which the other strains did not: an ability to produce STb (Table 2; Fig. 1). This contrasted with the results in intact neonatal piglets, in which strains 431 (heat-stable toxin a [STa] only) and 987 (STa and STb) both attracted neutrophils. However, we wanted to determine whether STb could be one factor which attracts neutrophils. For that reason, we obtained the E. coli strains HB101 and HB101(pRAS-1) and tested the ability of sterile culture supernatants of these strains (STb preparations) to attract neutrophils into ligated intestinal loops.

Four of the six pigs tested responded to STb enterotoxin, as noted by the presence of larger amounts of fluid in these loops after 4 h of incubation ($P < 0.01$) (mean + standard error for 10.6 ml + 1.15 for the E. coli HB101 loops and 3.12 ml + 2.24 for the E. coli HB101(pRAS-1)

Table 4. Neutrophils in ligated intestinal loops of two pigs used to compare effects of live and killed E. coli 263

Loop contents	n	Mean number of neutrophils per ligated loops + SE
Control	3	79,730 + 58,507 [a]
Killed 263	5	260,049 + 86,718 [a]
Live 263	3	3,357,082 + 393,024 [b]

[a,b] Means with different superscripts are different, $P < 0.01$.

loops). In these four pigs, there was no relationship between neutrophil attraction into the lumen or wall of the intestine and the presence or absence of STb in a ligated intestinal loop (mean + standard error for percentage of neutrophils was 18.9 + 9.2 for BHI loops, 26.9 + 9.2 for E. coli HB101 loops, and 31.0 + 9.7 for E. coli HB101(pRAS-1) loops).

Discussion

We demonstrated that neutrophils entered the lumen of the small intestine of neonatal piglets when that intestine was intensively colonized with ETEC and that exposure to a nonenteropathogenic strain of E. coli did not attract similar numbers of enteroluminal neutrophils. Neutrophils with phagocytosed E. coli cells have been reported on the villi of neonatal piglets with colibacillosis [14,p.509]. Jubb and Kennedy also report a purulent intestinal content which is occasionally observed in piglets with colibacillosis [10,p.109]. However, this is the first experimental demonstration that when the small intestine of a neonatal piglet is colonized with E. coli cells (at least with these two strains), enteroluminal neutrophils are seen in significant numbers.

Although the small intestine of intact neonatal piglets is the most natural system in which to study this phenomenon, the ligated intestinal loop is better suited for dissecting out variables which could influence the attraction of neutrophils. Gut motility is not a factor when one is working with ligated loops, and it is possible to compare several different treatments within one animal.

It seems unlikely that there is any single bacterial attribute which determines elicitation of neutrophils. Many types of agents have been demonstrated to affect neutrophil behavior; these range from formyl-methionyl peptides to lipopolysaccharides to immune complexes [17]. The aim of our work with ligated loops was to attempt to identify one or some of the factor(s) which might attract neutrophils into the lumen of the intestine.

The ability to attract neutrophils into the lumina of ligated intestinal loops varied with the strain of E. coli. Strains 263, 1288, and 987 definitely attracted more neutrophils into the intestinal lumina than did the controls. These same three strains continued to attract neutrophils at 6 h postinoculation, when other strains did not.

The three strains which elicited the strongest luminal response in the ligated loops (strains 263, 1288, and 987) share one factor which the other strains do not (Table 2). This factor is STb. According to Bellamy and Nielsen [3], neutrophils could be elicited into the lumen if antigen was placed in the lumen and if the animal in question had specific circulating antibody. Possibly our pigs, which were all obtained from the same source, shared a similar history of antigen exposure. However, this work spanned 5 months, and it seems unlikely that this herd sustained continued exposure to STb but not to other antigens of E. coli. Furthermore, STb has minimal antigenicity [22]. Thus, immunity to STb seems unlikely to explain the neutrophil response to these strains.

The STb could conceivably attract neutrophils either directly or even indirectly, through subtle tissue damage. However, examination of porcine ligated intestinal loops exposed to supernatants of E. coli HB101 and HB101(pRAS-1) indicated that STb had no effect on the number of neutrophils attracted to the wall or lumen of the intestine. (Fluid secretion was comparable in these STb-positive supernatant loops and in loops from experiment 2 containing whole STb+ bacteria; STb concentrations in these loops were also judged to be comparable.) Furthermore, strain 431 (which does not produce STb) elicited neutrophils in intact neonatal piglets (Table 3). Thus, the enteroluminal neutrophil response to porcine ETEC cannot be explained by STb per se.

Two of the strains which attracted neutrophils so strongly (strains 263 and 1288) are labile toxin (LT) positive. We did not test for the role of LT in the attraction of enteroluminal neutrophils in loops. However, in vitro evidence indicates that LT suppresses chemotactic activity of neutrophils [4].

We also considered the possibility that bacterial adhesion to epithelium might be required, in view of the fact that strain 431 gave a positive response in neonates, in which it adheres, but was negative in older pigs, in which it does not adhere [18], and that the 987P and K88 antigens of the two strains that gave positive reactions in intestinal loops of older pigs mediate adhesion to pig intestine. We were unable to demonstrate any correlation between positive response and adhesion by examining histological sections from the loops. This could indicate that

adhesion is not required for a neutrophil response in the closed loop system, where bacteria can attain high numbers without adhesion, or it could indicate that the histological method used was not appropriate for differentiating between adherent and nonadherent bacteria. The role of adhesion in attracting neutrophils could perhaps be determined by using isogenic pairs (with and without the adhesins) of strains that do attract neutrophils.

The E. coli cells apparently had to be alive to attract neutrophils into the intestinal lumen. However, the strains which attracted more neutrophils into the lumina of loops did not achieve this by an exceptional proliferation of E. coli cells; strains which did not attract neutrophils grew just as rapidly as those that did.

How do the numbers of neutrophils we observed compare with numbers of neutrophils noted in other conditions or sites? If we examine the neutrophils elicited into ligated intestinal loops by E. coli 263 after 6 h of incubation, we find the gut fluid contains 460 neutrophils per microliter. A slightly younger pig has approximately 5,400 neutrophils per microliter in the blood [19, p. 201]. Bellamy and Nielsen were able to attract nearly four times as many neutrophils into intestinal loops as we were [3]. The mammary gland is another mucosal surface on which neutrophils are known to be present in large numbers. Normal cows in the latter part of lactation have neutrophil counts as high as 1,000/microliter [6]. Neutrophil counts in excess of 3,600/microliter occur in milk from infected glands [7, pp. 367;8]. Because all these

measurements represent static measurements of dynamic neutrophil populations, they may not mean as much as we would like them to. That is, a study of gut neutrophil kinetics would have more meaning than does this type of measurement [16].

In response to Sellwood's question, we have shown that neutrophils are present in increased numbers in the intestinal lumina and walls of neonatal piglets suffering from colibacillosis. Using the ligated intestinal loop model in older pigs, we found that attraction of neutrophils into the intestine was influenced by the strain of E. coli inoculated into the loops. At this point, we have found no single attribute of an E. coli strain which is correlated with its ability to attract neutrophils. In the future, we hope to elucidate the abilities these enteroluminal neutrophils have with respect to bacterial killing.

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GENERAL DISCUSSION

This thesis has touched on two principal phenomena within the area of intestinal pathology and defense. Enteroluminal neutrophils may be an important protective mechanism within the intestine [8,35,84]. Enterotoxins elaborated by Escherichia coli are undoubtedly a major cause of intestinal malfunction in both humans and animals [25,31,89]. Rather than simply repeating the conclusions of the two papers included within this thesis, it is more interesting to look ahead to future developments in these areas.

Enterotoxins have been the subject of investigations by many people. People in the fields of pathology, physiology, biochemistry, internal medicine, and molecular genetics have been active in this research for many years. A partial comprehension of molecular-level mechanisms has been achieved regarding cholera toxin, E. coli heat-labile toxin, and E. coli heat-stable enterotoxin a [25,32,76,89]. The E. coli heat-stable enterotoxin b (STb) has been more difficult to work with. Although the pig was known to respond to STb with intestinal secretion some pigs did not respond to STb. Even in responding pigs, different ligated loops may or may not respond to STb with secretion. This highly variable response to STb has made it a difficult toxin to investigate. The finding that STb is susceptible to degradation by intestinal proteases [100] should make the subject of STb toxicity a more attractive area of research. Attempts to purify the toxin can proceed more rapidly as the assay for its presence is more reliable.

Although enteroluminal neutrophils have also been the subject of inquiry for many years [1,8,10,81,84], a comprehensive view of these cells has yet to emerge. Work by Bellamy which appears to be reliable indicates that attraction of neutrophils into the lumen of the intestine can be immune-mediated, largely through humoral mechanisms [8]. Unfortunately, Bellamy's work has not been repeated although attempts to repeat it have been made (R. Rose, NADC, Ames, Iowa, unpublished data; J. Kelrby, Dept. of Animal Husbandry, University of Bristol, Bristol, England, personal communication). Perhaps the well-known phenomenon of intestinal immune tolerance has thwarted attempts to repeat Bellamy's work, or perhaps the mode of the intestinal immune response to an antigen determines whether or not a recognized antigen can attract neutrophils into the lumen of the intestine.

One impediment to working with enteroluminal neutrophils is that they are difficult to quantify accurately. The intestinal lumen can be an inhospitable environment for cell survival, and it is difficult to know how many neutrophils have already entered the lumen and disintegrated. Use of neutrophil biochemical markers would seem to be attractive and has been used to try to quantify enteroluminal neutrophils [9,10] although the molecules chosen (lactoferrin and lysozyme) are not necessarily specific for neutrophils. Even if a more specific biochemical marker were chosen, however, proteases within the intestine might well degrade significant amounts of the molecule in question. A radioactive label such as ^{111}indium has been used to monitor fecal excretion of intestinal

granulocytes in Crohn's disease [82]. Indium-111-labeled neutrophils have been shown to be structurally intact and to have normal *in vitro* locomotion and bactericidal activity [108].

An analogy can be drawn between work on STb and work on enteroluminal neutrophils: one needs to know whether one has STb in an intestinal loop, and one needs to be able to quantify enteroluminal neutrophils. Protease inhibitors in loops have improved the STb assay. Indium-111-labeled neutrophils could remove much of the subjectivity that has plagued work on enteroluminal neutrophils.

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ACKNOWLEDGMENTS

I am grateful to Dr. Harley W. Moon for the opportunity to work at the National Animal Disease Center during the time that this research was initiated and completed. Dr. Lawrence H. Arp was very helpful in helping me negotiate various graduate college requirements and was the source of pertinent and helpful advice; he is much appreciated. The technical assistance of Jayme Reed and Ruth Willson was of a high quality.